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RAPIDLY INDUCED CHANGES IN THE LEVEL OF NON-SPECIFIC IMMUNITY IN LABORATORY ANIMALS

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MANY species of Gram-negative bacteria are rapidly killed when incubated with fresh normal animal or human serum. There is often, however, considerable inter-strain variation in susceptibility to this killing action within any one bacterial species, and it has been suggested (Maaløe, 1948; Rowley, 1954) that this variation may account for inter-strain differences in virulence. The necessary presence in the serum of complement and magnesium ions is established but apart from this the chemical mechanisms involved in this bactericidal action are entirely unknown. It was recently suggested that the bactericidal action of serum involves a reaction between serum and some surface substrate of the organism (Rowley, 1955). Support for this suggestion was given by the finding that the injection into mice of a crude preparation of the cell walls of *Bacterium coli* produced a rapid and striking increase in the susceptibility of the animals to a subsequent challenge by *Bact. coli*. This increased susceptibility lasted only a few hours and was followed 24 hours later by a state of immunity in the animals towards challenge by *Bact. coli* which lasted for about a week. Simultaneously with these changes in susceptibility there were corresponding changes in the bactericidal power of the serum. In view of these observations, attempts have been made to obtain in a more purified state the active principle present in the crude cell wall preparations. This has led to the isolation of a lipopolysaccharide, which probably contains the ultimate substrate for the bactericidal reaction in the serum.

MATERIALS AND METHODS

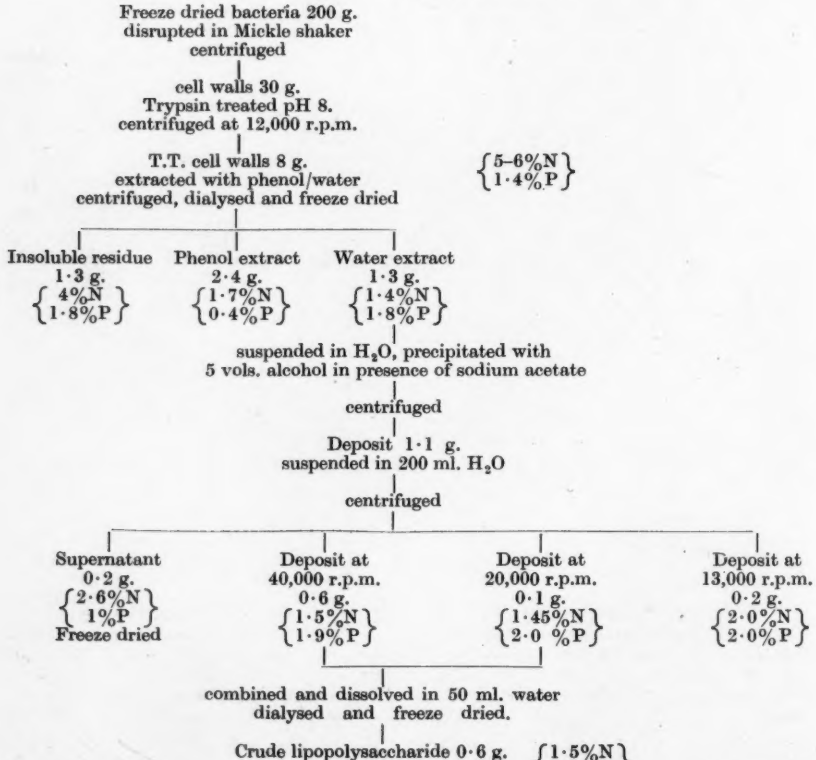
Strains.—The following strains were used :—
Bact. coli 2380, 2206, 75, 145, K12 (Rowley, 1954).
Salmonella paratyphi B—strain Kroger.
Salmonella typhi murium M206.
Proteus vulgaris, Pseudomonas pyocyanea, Klebsiella pneumoniae isolated from routine culture plates.
Corynebacterium diphtheriae strain P.W.8.
Bacterial extracts.—Bacterial cell wall preparations, made by disintegrating bacteria in a Mickle shaker, were treated with trypsin as previously described (Rowley, 1955). The

preparations from *C. diphtheriae*, *S. typhi murium* etc. were used without further purification, but those from *Bact. coli* (2380 and K12) were subjected to further extraction processes. Two methods were used :

(a) *Diethylene glycol extraction* was by the method described by Morgan (1937). This treatment resulted in 90 per cent by dry weight of the cell wall becoming soluble. After removing the solvent by dialysis, the extract was freeze-dried.

(b) *Extraction with* 90 *per cent phenol water at* 65° after the method of Westphal, Lüderitz and Bister (1952) separated the cell walls into three fractions : (*a*) phenol soluble, (*b*) water soluble, (*c*) insoluble residue. The water-soluble fraction was further purified by precipitation with 90 per cent alcohol in the presence of sodium acetate, the precipitate was suspended in water and centrifuged successively at 13,000 r.p.m. and 20,000 r.p.m., in a Serval high speed angle centrifuge, the supernatant was recentrifuged at 40,000 r.p.m. in a Spinco ultracentrifuge. The deposits at 20,000 and 40,000 r.p.m. which proved to possess the greatest biological activitiy, were mixed and re-suspended in water before dialysis and freeze-drying. A chart of the purification process together with the nitrogen and phosphorus analyses of the various fractions is shown in Table I.

TABLE I.—*Preparation of Crude Bacterial Lipopolysaccharide (after Westphal-Lüderitz)*



For nitrogen and phosphorus analyses the methods described by Kabat and Mayer (1948) were used.

In vitro *bactericidal tests*

Dilutions of the serum were made, using minimal medium (Davis and Mingioli, 1950) as diluent in sterile test tubes, the final volume in each tube being 1 ml.; this was incubated for ½ hr. to reach 37° and 0·02 ml. of a dilution of the culture added, such that each tube contained approximately 1,000 organisms. Counts were made on 0·1 ml. samples from each tube spread on nutrient agar plates at times zero, 20 min., 40 min., and 80 min. from the start, during which time the tubes were kept at 37°. Fig. 1 shows a typical result obtained with fresh rat serum and *Bact. coli* K12. Duplicate counts did not differ by more than 20 per cent.

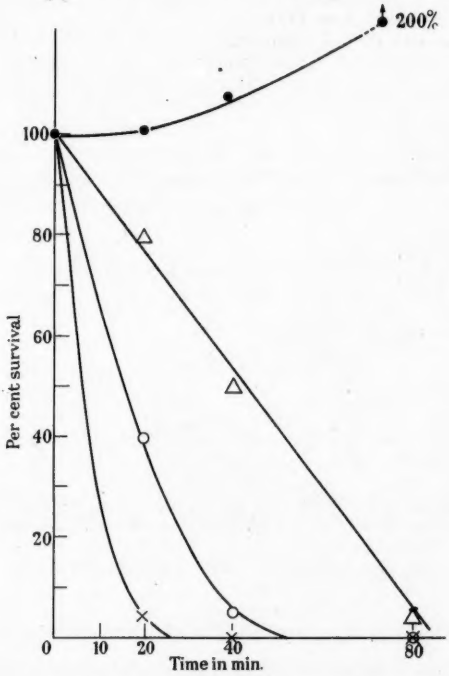


FIG. 1.—Survival of *Bact. coli* K12 in dilutions of rat serum at 37°.

× = neat rat serum and 1/5.
○ = dilution 1/10.
△ = dilution 1/40.
● = diluent (minimal medium).

The inhibitory effect of various bacterial fractions on the bactericidal reaction was shown by incorporating varying amounts of the fraction into a duplicate set of tubes, together with the serum dilutions and organisms as above. Fig. 2 shows how the addition of 0·1 mg. trypsin-treated cell walls from *Bact. coli* (2380) prevented the killing of *Bact. coli* (2206) by fresh guinea-pig serum. This test was done with all the bacterial extracts preliminary to *in vivo* tests.

In vivo *tests*

All materials injected into mice were in a volume of 0·2 ml. and with intravenous injections were given into one of the tail veins; the challenge organisms were always given by the intraperitoneal route suspended in 1·7 per cent hog mucin and 0·4 per cent charcoal (Rowley,

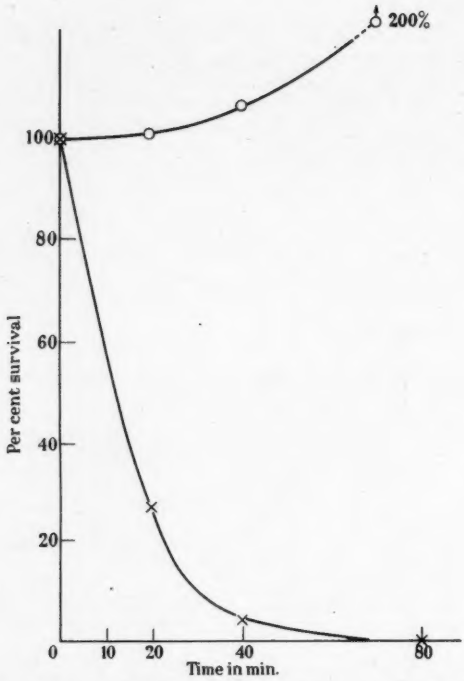


FIG. 2.—Survival rate of *Bact. coli* 2206 in guinea-pig serum with and without the simultaneous addition of 0·1 mg./ml. *Bact. coli* 2380 cell walls.

× = guinea-pig serum alone.
○ = same serum plus 0·1 mg./ml. *Bact. coli* 2380 cell walls.

1954). For viable counts the drop method of Miles and Misra (1938) was used in triplicate on the injected dilutions.

The cumulative results from about 500 mice with each bacterial strain have been used to assess the LD_{50} values by the method of Reed and Muench (1938). These are :

Bact. coli	75 and 145	10^3	organisms
,,	2380	10^4	,,
,,	2206	10^7	,, (Rowley, 1954).

RESULTS

Effects of Bacterial Cell Walls on the Susceptibility of Animals to Bact. coli.

Trypsin-treated cell walls of *Bact. coli* 2380 were injected intraperitoneally (i.p.) into mice (0·1 mg./mouse) and the mice challenged within 1 hr. with various strains of *Bact. coli*. Table II shows the results, and it is evident that the mice given cell walls were, at this time, much more susceptible to the experimental infection than were the normal control mice.

On the other hand, when mice were challenged at 24 or 48 hr. after giving an injection of cell wall preparation, a quite different state of affairs existed. Table

TABLE II.—*Effect of Injecting Bacterial Cell Walls on the Immediate Susceptibility of Mice to* Bact. coli *Challenge*

Injected with cell walls.	Time before challenge.	No. of organisms in challenge dose.	Deaths. Treated.	Deaths. Controls.
Bact. coli 2380 0·05–0·1 mg. i.p.	30 min.	*Bact. coli* 2206 2×10^5	19/21*	4/15*
		Bact. coli 2206 2×10^4	16/21	2/15
		Bact. coli 2206 4×10^3	18/21	0/15
Bact. coli K12R 0·2 mg. i.p.	30 min.	*Bact. coli* 2206 2×10^4	4/6	0/6
		Bact. coli 2206 4×10^3	3/6	0/6

* Numerators = numbers of deaths. Denominators = total numbers challenged.

III summates many such experiments, the results of which were completely opposite to those in Table II since the treated mice were now much more resistant than the untreated control animals.

TABLE III.—*Effect of Injecting Trypsin-treated Cell Walls of* Bact. coli 2380 *on the Susceptibility of Mice to* Bact. coli *Challenge 24–48 hr. Later*

Dose of cell walls.	Route of injection.	Numbers of organisms in challenge dose. 10^7.	10^6.	10^5.	Percentage deaths.
		Strain 145			
0·1 mg.	i.p.	4/21*	2/21	2/21	12
0·05 mg.	i.v.	6/27	3/27	2/27	13
Controls, Strain 145	—	32/36	30/36	26/36	81
		Strain 75			
0·1 mg.	i.p.	4/11	0/12	0/12	11
Controls, Strain 75	—	12/12	12/12	12/12	100
		Strain 2380			
0·1 mg.	i.p.	3/12	0/12	0/12	8
0·05 mg.	i.v.	0/5	0/5	0/3	0
Controls, Strain 2380	—	9/9	9/9	7/9	93

* Numerator = numbers of deaths. Denominator = total numbers challenged.

This rapidly developing immunity had disappeared by about seven days (see also Field, Howard and Whitby, 1955), its duration depending to some extent on the dose of cell walls initially injected. The antigenic structure of the challenge organism was of no consequence ; from Table III it is seen that cell walls of *Bact. coli* 2380 gave good protection against either the homologous organisms or against two other strains, *Bact. coli* 75 and 145. The latter strains were not typable with any of the usual *Bact. coli* antisera whereas strains 2206 and 2380 belong to type 0111 B4.H12. Moreover, mice immunised with living cells of *Bact. coli* 2206 and challenged 12 days later with strains 2380, 145 and 75 were found to be immune to the antigenically homologous strain 2380 but normally susceptible to strains 145 and 75 (Table IV).

Exactly similar changes in susceptibility to *Bact. coli* challenge could be brought about by the previous injection of the cell walls of *S. typhi murium*, *Ps. pyocyanea*, *P. vulgaris* or *K. pneumoniae*.

TABLE IV.—*Cross Protection Tests with Mice Immunised I.P. with* 10^7 *Organisms of Living* Bact. coli 2206 *and Challenged with Different Strains after* 12 *Days*

Challenge strain.	Number of organisms in challenge dose + 1·75 per cent hog mucin.		
	10^6.	10^5.	10^4.
Bact. coli 145 . . .	3/3	2/3	3/3
Bact. coli 75 . . .	3/3	2/3	3/3
Bact. coli 2380* . .	0/3	0/3	0/3

* Strains 2206 and 2380 are antigenically similar.

This early immunity is not associated with any detectable change in the level of agglutinating antibody to the challenge organisms, nor with the appearance of measurable amounts of whole haemolytic complement in the serum of protected mice.

Although most of the present experiments have involved mice, the same biological changes occurred in other animals. It is not possible to demonstrate protection against *Bact. coli* in rats, since normal animals can withstand 10^7 organisms in mucin ; challenge within a short time of injecting *Bact. coli* cell walls, however, resulted in an increase of susceptibility (Table V). Guinea-pigs being moderately susceptible to challenge with *Bact. coli* + mucin behaved very much like mice in their changes in immunity following administration of trypsin-treated cell walls.

TABLE V.—*Changes in Susceptibility to* Bact. coli 145 *of Guinea-pigs and Rats following Injection of* Bact. coli 2380 *Cell Walls.*

Dose of cell walls.	Time before challenge.	Numbers of organisms in challenge dose.			
		10^7.	10^6.	10^5.	10^4.
Guinea-pigs					
1 mg. i.p. . . .	30 min. .	—	6/6	4/6	5/6
None (controls) . .	— .	—	3/4	1/4	0/4
1 mg. i.p. . . .	48 hr. .	0/3	0/3	0/3	—
1 mg. i.p. (controls) .	No challenge 0/12				
Rats					
0·2 mg. i.v. . . .	30 min. .	—	8/8	5/8	5/8
None (controls) . .	— .	—	0/4	0/4	0/4
0·2 mg. i.v. (control) .	No challenge 3/12				

It should be noted that the cell wall material produced early immunity whether given i.p. or i.v. ; on the other hand, substances such as starch or broth which have been shown to produce a transient immunity (Pfeiffer and Issaeff, 1894), or hog mucin, must be given i.p. in order to be effective. When these substances were given intravenously there was no effect on the subsequent i.p. challenge (see Table VI).

Effect of Purified Extracts on Susceptibility to Bact. coli *in Mice.*

All the Gram-negative bacterial cell walls which were examined contain material which produced the early changes in immunity described above. The active material could be extracted into diethylene glycol or into the water phase

TABLE VI.—*Effect of Injecting Various Materials on the Susceptibility of Mice to* Bact. coli 145 *Challenge* 48 *hr. Later.*

Material.	Dose (mg.).	Route.	Challenge dose of *Bact. coli* 145 10^6	10^5	10^4	Total deaths.
Starch	0·1	i.p.	0/3	0/3	2/3	2/9
,,	0·1	i.v.	3/3	3/3	3/3	9/9
Hog mucin	3	i.p.	1/3	0/3	0/3	1/9
,,	3	i.v.	3/3	3/3	3/3	9/9
Bact. coli K12R T.T. cell walls	0·05–0·1	i.v.	4/9	0/9	0/9	4/27
Controls	—	—	15/15	15/15	12/15	42/45
C. diphtheriae T.T. cell walls	0·2–0·5	i.v.	7/15	4/15	3/15	14/45
Ditto	0·1	i.v.	3/3	0/3	1/3	4/9
,,	0·04	i.v.	3/3	3/3	3/3	9/9
Controls	—	—	12/12	10/12	9/12	31/36

of a phenol/water mixture as described earlier. This extracted material contained 2·0 per cent phosphorus and 1·5 per cent nitrogen; it was very stable at 100°, the biological activity being little impaired after 7 hr. boiling in water. These properties were similar to those of the lipopolysaccharides of Gram-negative bacteria obtained by similar extraction procedures by Westphal and Lüderitz (1954).

Table VII shows that the minimum amounts of the present lipopolysaccharide material required to protect mice against subsequent challenge was of the order of 1 μg. per 20 g. mouse, compared with a value of approximately 10 μg. using the most active purified lipopolysaccharides of Westphal. This difference may have been due to the bacterial strain from which the lipopolysaccharides were derived, since the lipopolysaccharides kindly supplied by Professor O. Westphal from twelve different Gram-negative bacteria varied considerably in protective ability.

TABLE VII.—*The Susceptibility of Mice to Challenge by* Bact. coli 145, 48 *hr. after the I.V. Injection of Purified Lipopolysaccharide from* Bact. coli 2380

Dose of lipopolysaccharide injected.	Challenge dose of *Bact. coli* 145 10^6.	10^5.	10^4.	Percentage deaths.
0·05 mg.	4/21	1/21	1/21	9
0·01 mg.	15/21	6/21	2/21	36
0·001 mg.	10/15	5/15	4/15	42
Controls	15/15	11/15	13/15	86

The question of the exact identity of the bacterial substrate is still open. There are several reasons for suspecting that the whole lipopolysaccharide molecule may not be the ultimate substrate; for example, a sample of "degraded" polysaccharide from *Shigella shigae* (Davis, Morgan and Record, 1955) kindly supplied by Dr. W. T. J. Morgan produced immunity 48 hr. following injection of 0·1 mg./mouse, while the Friedlander polysaccharide of Ginsberg, Goebel and Horsfall (1948) was similarly active at a dose of 0·1 mg./mouse. Fairly active materials have also been extracted from Gram-positive organisms (see Table VI).

Toxicity of fractions

Since the various fractions increased the susceptibility to *Bact. coli* infection, it was important to determine their toxicity. From Table II it can be seen that of the cell walls of *Bact. coli* 2380 approximately 1/20 of an LD_{50} dose produced an increase in susceptibility to immediate challenge. It does not seem likely that this immediate depression in immunity can be due to the summation of two sub-lethal effects. The early stimulation of immunity has been produced by less than 1/100 of an LD_{50} dose (Table VIII).

TABLE VIII.—*Toxicity for Mice of Various* Bact. coli *Fractions*

Material.	Toxic dose LD_{50}/20 g. mouse. (mg.).	Minimum dose per mouse giving 48 hr. protection. (μg.).
Bact. coli 2380 trypsin-treated cell walls	1	10
Bact. coli 2380 crude lipopolysaccharide	0·3	1
Bact. coli 2380 residue from phenol/H_2O extraction	4	10
Bact. coli K12 trypsin-treated cell walls	10	50
Bact. coli K12 crude lipopolysaccharide	3	10
Bact. coli K12 residue from phenol/H_2O extraction	10	50

Changes in the bactericidal property of serum from animals injected with cell wall fractions

Experiments were made to investigate the bactericidal properties of serum from animals treated with bacterial extracts. Since mouse serum is non-bactericidal when drawn it was not possible to determine with this serum whether changes occurred. Changes were however seen in guinea-pig and rabbit serum following injection of lipopolysaccharides. In one experiment three guinea-pigs were bled and their sera pooled; each was then injected i.p. with 5 mg. of cell walls of *Bact. coli* (2380) suspended in saline, and each was bled after 1 hr. and 24 hr. The pooled serum from the untreated animals was able to destroy 1,000 cells/ml. of *Bact. coli* (2206) when diluted 1/2 with saline; 1 hr. after the injection of cell walls the pooled serum was not bactericidal even when undiluted, whereas after 24 hr. the pooled serum was active when diluted 1/8 in saline.

Fig. 3 presents data from a similar experiment in which the guinea-pigs were injected i.p. with 5 mg. of the cell walls of *P. vulgaris*.

Relationship between cell wall activity and virulence

It has been suggested (Rowley, 1954) that the variations in virulence for mice from one strain of *Bact. coli* to another may depend on the susceptibility of the strains to lysis by complement-containing sera. Such variations must depend either on different quantities or accessibility of the bacterial substrate between the strains. When the cell walls were prepared from two such strains, *viz.*, *Bact. coli* 2380 (virulent) and *Bact. coli* 2206 (avirulent) it was found that those of the virulent strains were more active in protection experiments, suggesting that the virulent strain had a higher content of the active lipopolysaccharide. This was further supported by determining the minimum amounts of these bacteria and their cell walls which would inactivate the C'3 content of 1 ml. of human serum.

I am grateful to Professor L. Pillemer and Miss Leona Wurtz for these determina-

tions. Table IX shows that the virulent strain was consistently more active in this respect.

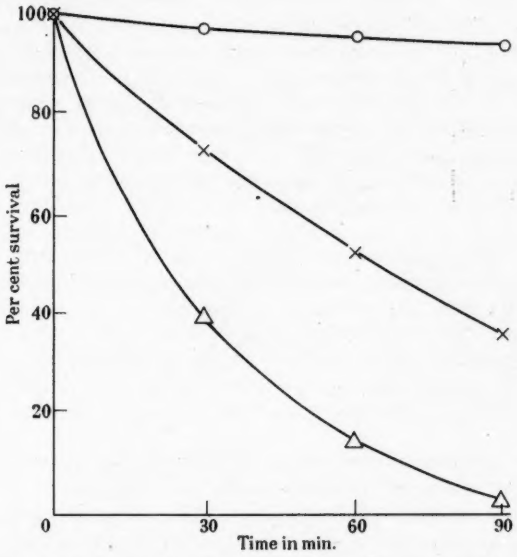


FIG. 3.—Bactericidal power toward *Bact. coli* 2206 of pooled guinea-pig serum drawn before, and at intervals after the injection of *P. vulgaris* cell walls. Each of 4 guinea-pigs injected i.p. with 5 mg. *P. vulgaris* cell walls. Serum diluted 1 vol. in 2 vols. minimal medium.
× = before injection with cell walls.
○ = 80 min. after injection.
△ = 24 hr. „ „

TABLE IX.—*Comparison between* Bact. coli 2380 (*Virulent*) *and* 2206 (*Avirulent*) *for Their Capacity to Bind the Third Component of Complement in Human Serum*

Substance.	Amount in mg. required to inactivate C'3 in 1 ml. human serum. Av.	V.
Living bacteria	2·0	<1·0
Killed bacteria (60° for ½ hr.) . . .	>5·0	5·0
Cell walls	>2·0	0·2

If the content of bacterial lipopolysaccharide is a determinant of sensitivity to the bactericidal power of serum, as the above suggests, then it is to be expected that changes in strains from S→R which are known to cause a decrease in the amount of O antigen and hence of the associated lipopolysaccharide, will result in changes in the sensitivity of the strain to serum. The action of serum on six bacterial strains and on the rough mutants derived from them by growth in O antisera, was examined. In every case the rough strain was more rapidly killed; examples are shown in Fig. 4.

Another way of showing this increase in susceptibility is to determine the maximum dilution of the serum which will cause a standard amount of killing in a given time. In one such experiment the smooth strain of *Bact. coli* (2206)

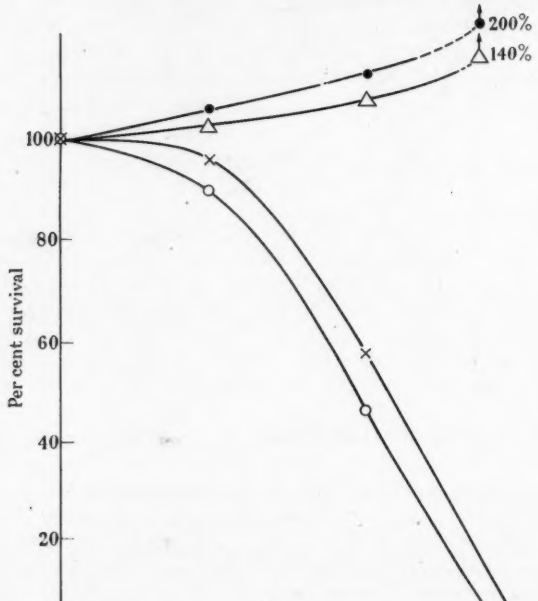


FIG. 4.—Survival rates of rough and smooth mutants during incubation in guinea-pig serum diluted 1 vol. in 4 vols. minimal medium.
● = *S. paratyphi* B (Kröger) smooth.
○ = *S. paratyphi* B (Kröger) rough.
△ = *Bact. coli* 2206 smooth.
× = *Bact. coli* 2206 rough.

showed 50 per cent survival after 90 min. at a dilution of guinea-pig serum of 1/2 in minimal medium, whereas a rough mutant of the same strain showed the same survival at a dilution of 1/16.

DISCUSSION

It has been shown that the early changes in the resistance of animals to infection with coliform organisms are accompanied by simultaneous variations in the bactericidal powers of the serum and the possibility exists that these bactericidal variations are the cause of the immunity effects. If this is so, the results obtained here are consistent with the hypothesis that the bacterial lipopolysaccharide contains the reactant for the bactericidal reaction. In this event the injection of this bacterial substrate rapidly inactivates the presumed enzyme system in serum, which in a few hours is replaced and the loss is over-compensated

in the manner of an adaptive enzyme. In support of this view is the finding that the early immunity can be transferred to some extent by the serum of protected animals to normal mice.

Also consistent with this interpretation is the finding that rough strains are much more sensitive to the bactericidal system than are the smooth strains from which they were derived. The rough strains contain less O antigen and therefore less of the particular lipopolysaccharide material which is a component of it. This smaller amount of substrate in rough strains is analogous to the smaller amount in one smooth avirulent strain as compared to an antigenically similar virulent strain. It seems likely that the speed with which the small quantities of reactant present in rough or avirulent organisms can be attacked is a major determinant of their lack of virulence.

The question arises whether the virulence-enhancing powers of mucin can be due to an interference with the humoral defences of the host such as that described here. Hog mucin is known to be anti-complementary in relatively large doses (Lambert and Richley, 1952); nevertheless there is one piece of evidence against a humoral action. If hog mucin is given intravenously and the organisms given ntraperitoneally there is no enhancement of virulence (personal observations); moreover intravenous injection of hog mucin does not produce any early non-specific immunity.

The effect of the lipopolysaccharide is further distinguishable from that of hog mucin by the finding that injection of active bacterial substrate fractions either i.p. or i.v. together with *Bact. coli* but without mucin does not have any marked virulence-enhancing powers. It seems that hog mucin inhibits the action of the cellular defences and resolves the struggle between bacteria and host into one with the humoral defences (Olitzki, 1948), and it is for this reason that the changes in the bactericidal powers of the serum are so markedly paralleled by the degree of resistance to this artificial infection.

Our understanding of the nature of the bactericidal reaction in normal serum has been considerably advanced recently by Pillemer, Blum, Lepow, Ross, Todd and Wardlaw (1954); they have shown that besides complement and magnesium ions, a protein factor named properdin is necessary for bactericidal activity. This factor, properdin, is removed from serum *in vitro* by incubation in the presence of a yeast cell wall preparation called zymosan. It has been found that i.v. injection of zymosan into mice, rats or guinea-pigs produces very similar changes in early immunity to those produced by cell walls of Gram-negative bacteria (Rowley 1955). Pillemer and Ross (1955) have also shown that following the injection of zymosan or *Bact. coli* cell walls into rats or mice there is a rapid fall in the serum properdin which is rapidly replaced and within 24–48 hr. reaches a level often considerably higher than normal; this increased level falls down to normal around the eighth day.

Owing to the scarcity of purified properdin it has not so far been possible to prove conclusively that these early changes in immunity are a result of the simultaneous changes in properdin levels. However, three mice each injected with 20 units of properdin survived immediate challenge with *Bact. coli* (145) whereas the three control animals died; this slight evidence may be coupled with the finding that rat serum which is rich in properdin will effectively protect mice against *Bact. coli* challenge.

The evidence is in favour of the early immunity effects being due to changes

in available active properdin, these changes being induced by contact with the bacterial substrate which itself appears to be a part of the very high molecular lipopolysaccharide fraction in bacteria.

SUMMARY

The injection of Gram-negative bacterial cell walls into animals causes rapid changes in the susceptibility of the animals to subsequent infection with *Bact. coli* + mucin. During the first hour the animals are much more susceptible, but after 24 hr. a state of resistance develops.

The active material in the bacterial cell walls has been shown to occur in the lipopolysaccharide fraction. After injection of this active material into animals there is a rapid decrease followed within 24 hr. by an increase in the bactericidal powers of the animals' serum. It is suggested that the changes in bactericidal power of the serum are the cause of the changes in the immune state of the animal.

One smooth virulent strain of *Bact. coli* has been shown to have a higher content of active material than one smooth avirulent strain of the same antigenic type. Rough strains (like smooth avirulent strains) are more sensitive to the killing action of serum than are the smooth parent strains from which they were derived.

The possible relationship between these immunity effects and the concurrent changes which occur in serum properdin levels is discussed.

I am indebted to Professor L. Pillemer of Western Reserve University, Cleveland, for materials and valuable discussions, to Dr. H. Ginsberg for the Friedlander polysaccharide, to Professor O. Westphal of the A. Wander Institute, Säckingen, Germany for samples of purified lipopolysaccharides and to Dr. E. Kröger, Hygiene Institut, Göttingen, Germany, for various rough and smooth pairs of bacteria. I wish to thank the Medical Research Council for a grant toward the cost of animals. The excellent technical assistance of C. R. Jenkin is gratefully acknowledged.

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BLOCKADE OF SKIN PREPARATION IN THE SHWARTZMAN PHENOMENON BY MEANS OF HIGH-POLYMER SACCHARIDES

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ABILITY to inhibit skin preparation in the local Shwartzman phenomenon has been shown to be common to a variety of agents which cause leucopenia and depress acute inflammatory response, namely an endotoxin of Gram-negative bacteria (Ogata, 1936), nitrogen mustards, benzol (Becker, 1948; Schlang, 1950, 1952; Stetson and Good, 1951; Bennet and Cluff, 1952), and X-ray irradiation (Becker, 1948). It has been shown in particular that the protective effect of nitrogen mustard in the Shwartzman phenomenon is correlated with its leucopenogenic activity. When the femoral marrow of animals given mustard into a marginal ear vein had been protected by clamping the abdominal aorta, both leucopenia and the blocking effect of the nitrogen mustard on skin preparation failed to occur. These and similar findings have given rise to the idea that induction of skin reactivity in the local Shwartzman phenomenon depends on infiltration of the prospective site by granulocytes during skin preparation (Stetson and Good, 1951).

High polymer saccharides (levan and dextran) circulating in the blood suppress diapedesis (Shilo, Wolman and Hestrin, 1954; Shilo, Wolman and Wolman, 1956). These compounds markedly dimish capillary permeability (Davies, Shilo and Hestrin, 1955); furthermore they produce a severe though transitory leucopenia (Leusen and Essex, 1953; Bennet, 1952; unpublished experiments). If the hypothesis of Stetson and Good (1951) is valid, levan and dextran by preventing the accumulation of leucocytes at a skin site of toxin injection should block preparation. Experiments to test this inference have been performed.

MATERIAL AND METHODS

Toxin.—Cells of *Salmonella typhi* 0901, harvested from the surface of nutrient agar after 48 hr. growth, were washed, suspended in saline solution and disintegrated by shaking with Ballotini beads in a Mickle tissue disintegrator. After the débris had been removed by centrifugation, the fluid was sterilised by passage through a sintered glass filter. The sterile fluid ("toxin") was stored at − 20°.

Polymers.—Native levan was isolated from sucrose cultures of *Aerobacter levanicum*. The purification procedure and properties of this fructosan have been described (Davies *et al.*, 1955). Native dextran and fractions of defined molecular weights prepared from a partial hydrolysate of the native polymer were kindly donated by Dr. J. Ungar (Glaxo Ltd). A polyvinylpyrrolidone preparation (PVP K90) of high molecular weight was provided by Dr. D. B. Witwer (National Aniline Co.). Native dextran (CSC), clinical dextran (CSC) and several dextran fractions (NRC fractions) with molecular weights intermediate between native and clinical dextran were kindly provided by Dr. H. E. Staveley (Commercial Solvents Corp.).

Preparation of skin.—The abdomen of mongrel rabbits (1·5–2·0 kg.) was shaved with an electric clipper and depilated with barium sulphide paste. Twenty-four hours later, a graded series of toxin dilutions (60, 30, 6, and 3 Shwartzman skin-preparing units (Shwartzman, 1937)) in 0·1 ml. saline was injected intradermally.

Evocation of Shwartzman phenomenon.—Macromolecular substances, *e.g.*, acacia gum and dextran, are known to be efficient evoking agents (*cf* Bennet, 1952). Native levan, also, was found by us to possess this property. The haemorrhagic reaction which it evoked at prepared skin sites exceeded in extent and severity that produced by typhoid "toxin" injected intravenously. Levan (100 mg./kg.) was injected intravenously as a 3 per cent solution in saline 20–22 hr. after skin preparation. Under these conditions, typical haemorrhagic necrosis was evoked at all prepared skin sites in each of 12 rabbits of a control group. The minimal provoking dose of native levan was found to be of the order of 20 mg./kg.

Administration of polymers.—Polymers were given as a single intravenous dose (100 mg./kg.) at a concentration of 3 per cent in saline 20–30 min. prior to skin preparation.

Evaluation of skin reaction.—Necrosis at a competent skin site appeared within 60 min. of the provoking injection and reached the maximum intensity after 3½–4 hr. The longest axis (D) of the necrotic area and an axis (d) at right angles to it were then measured. The mean diameter of the necrotic area was calculated as $\sqrt{Dd}$.

RESULTS

Skin preparation was blocked completely or nearly so by both native levan and native dextran. Dextran fractions of molecular weights 130,000 and 500,000 caused a reduction of necrosis evoked at the sites of high toxin dosage (60 and 30 skin-preparing units), whereas clinical dextrans (molecular weight 60,000–70,000) and a polyvinylpyrrolidone preparation (molecular weight 350,000) failed to affect the phenomenon visibly. A numerical summary of all the data is presented in the Table.

TABLE.—*Effect of Different Polymers on Skin Response in the Local Shwartzman Phenomenon*

Polymer (100 mg./kg.) was injected 20–30 min. before intradermal injection of toxin. The evoking agent (levan 100 mg./kg.) was injected intravenously at 20–22 hr.

Toxin dose (skin preparing units).	Diameter of necrosis.*							
	Saline.	PVP K90 350,000.	Dextran C.S.C. 60,000.	Dextran Pharmacia 70,000.	Dextran Glaxo 130,000.	Dextran Glaxo $0{\cdot}5\times10^6$.	Dextran† Glaxo 5×10^6.	Levan‡ 10^7–10^8.
60	14±4	15±6	13±6	13±9	9±5	7±4	1±1	2±2
30	11±5	12±2	12±6	11±7	4±3	5±4	1±1	0
6	7±4	7±3	7±4	6±5	1±1	1±1	0	0
3	5±4	3±2	4±3	4±3	1±1	0	0	0
Number of rabbits	12	5	7	4	5	5	5	9

* Mean diameter (mm. ± standard deviation) per rabbit. Polymers are listed according to source. The adjoining figures give their approximate average molecular weights.

† 4 of 5 animals showed no necrosis at any site.

‡ 6 of 9 animals showed no necrosis at any site.

A striking correlation was found between ability to block skin preparation and leucopenic activity in the rabbit. Details of the experiments relating to the effect of intravenously administered polysaccharides (levans, dextrans) and polyvinylpyrrolidones of different molecular weights on the blood cell levels will be reported in detail separately. The trend of the findings is summarised in the accompanying Figure. All the polymers produced transient leucopenia, but the degree and duration varied characteristically with the molecular weight and chemical structure of the compound. The two substances (native levan, native dextran) which produced a severe leucopenia lasting for 2 hr. or more were also those which completely blocked skin preparation. On the other hand, the two

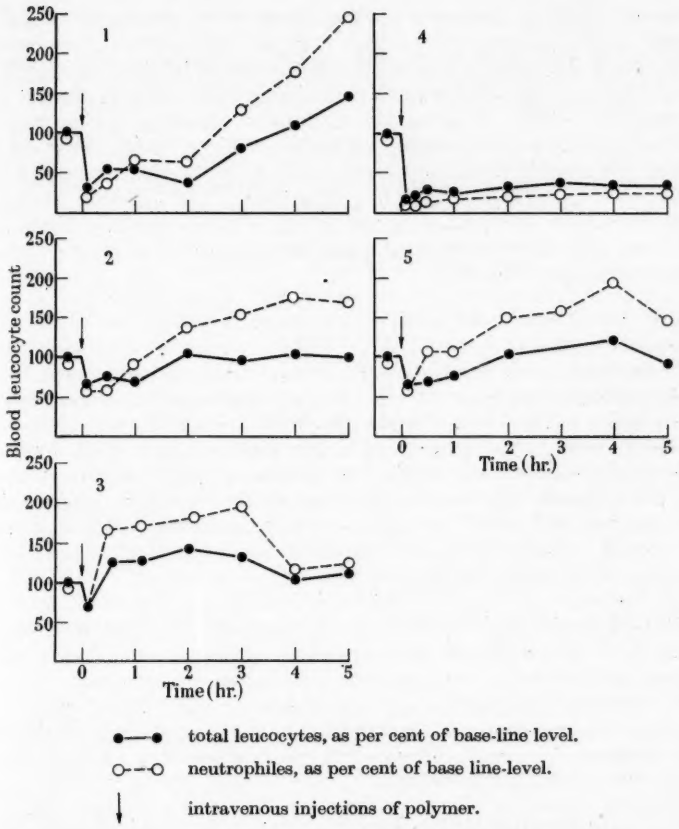

FIGURE.—Effect of polymers injected intravenously on the number of leucocytes circulating in the bloodstream.

At times indicated by the arrow (↓), rabbits received polymer (100 mg./kg. in 3 per cent solution) intravenously. Values plotted represent means of counts on 5 rabbits in each group: 1. Dextran (CSC, native), m.v. $\gtrsim 10^6$; 2. Dextran (CSC, NRC # 8), m.w. 410,000; 3. Dextran (CSC, clinical), m.w. 60,000; 4. Levan (*Aerobacter levanicum*, native), m.w. 10^7–10^8; 5. Polyvinylpyrrolidone (National Aniline Co., K90), m.w. 350,000.

substances—clinical dextran and polyvinylpyrrolidone K90—which produced only an evanescent mild leucopenia lasting less than an hour had no evident inhibitory activity on the Shwartzman phenomenon.

DISCUSSION

The present demonstration that skin preparation in the Shwartzman phenomenon is blocked by high-polymer saccharides which inhibit diapedesis is in good accord with the hypothesis (Stetson and Good, 1951) that leucocyte infiltration

of the prospective site is necessary to skin preparation. However, other possible explanations of the observed behaviour may not be ruled out entirely. It is pertinent to recall that high-polymer dextran and levan inhibit transfer of colloids as well as of cells from the blood to sites of skin injury (Davies *et al.*, 1955). Furthermore, a remarkable ability of levan and dextran to adsorb properdin has recently been discovered (Pillemer, Schoenberg, Blum and Wurz, 1955). It is conceivable that transfer of a soluble blood component such as properdin across the capillary endothelium could play a part in skin preparation. The question whether polysaccharides block skin preparation by inhibiting local escape of colloids from the vascular bed as well as by preventing diapedesis cannot be answered on the basis of the experiments here reported.

SUMMARY

Native *Aerobacter levanicum* levan administered intravenously to rabbits provoked the Shwartzman phenomenon at prepared skin sites. Skin reactivity to a provoking agent (native levan) in the Shwartzman reaction was not induced in animals which received a protective intravenous injection of high-polymer saccharide (native dextran or native levan) shortly before the injection of the preparing intradermal dose of toxin. The ability to block skin preparation decreased progressively with molecular weight within a polymer homologous series (dextrans). No blocking activity could be detected in clinical dextrans, *i.e.*. fractions of molecular weight $< 10^5$.

Intravenous injection of either native levan or native dextran produced an immediate and large though transitory fall in the number of the circulating leucocytes in the blood. The leucopenogenic activities of polymers and their ability to depress diapedesis and block skin preparation in the Shwartzman reaction are seen to be correlated properties.

The findings support the view that induction of skin reactivity in the Shwartzman phenomenon requires infiltration of the prospective site by leucocytes during the phase of skin preparation.

The technical assistance of Miss Ann Shapiro is acknowledged gratefully.

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THE COLONISATION OF IRRADIATED TISSUE BY TRANSPLANTED SPLEEN CELLS

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SPLEEN cells transferred from normal animals have been shown to counteract the lethal effects of ionising radiation in mice, by Jacobson, Simmons, Marks, Gaston, Robson, and Eldredge (1951). Jacobson (1952) postulated that these cells act on the host tissue through a humoral agent. This hypothesis is supported by the evidence of Hilfinger, Ferguson, and Riemenschneider (1953), and Cole, Fishler, and Bond (1953), that cell-free preparations of tissue are also able to counteract radiation injury. On the other hand, Barnes and Loutit (1954*a*, *b*) argued that the irradiated host tissue is repopulated by the transplanted cells.

The purpose of the present paper is to trace the fate of normal spleen cells injected into irradiated mice. In one series of experiments the injected cells were identified in the hosts by their iso-antigens, the donors and hosts being taken from two inbred strains of distinct iso-antigenic type. The method of identification depends on the ability of the iso-antigens in tissue cells to elicit transplantation immunity in a foreign host. At intervals after irradiation and injection, tissues of the host were transplanted into fresh hosts of the same strain. When the injected spleen cells or their descendants were still present in these tissues, they elicited in their fresh hosts a heightened resistance to grafts of their own antigenic type. For testing this graft resistance, an A-strain tumour (Sarcoma 1) was employed of which the growth in normal and immunised hosts had already been studied (Mitchison, 1955). In foreign hosts the tumour normally attains considerable size before regressing, while in hosts which have been previously immunised the growth is slight. This difference was used as the criterion of immunisation.

In a second series, injections were made of spleen cells which had been immunologically activated by intravenous injection of a bacterial antigen into the donors. The production of antibody by the cells in their hosts then served as a method of identification.

METHODS

Animals

The mice used in this study were of the A and CBA standard inbred strains.

Radiation

Five mice at a time were irradiated in aluminium cages, a uniform (± 3 per cent) X-ray beam being directed through the floor of the cage. The radiation was produced by a Westinghouse radiotherapeutic X-ray machine operated at 240 kV and 15 mA and had a Half Value Layer of 1·2 mm. Cu. The dose rate 1·5 cm. above the sawdust-covered floor was 43 R per min. Those mice which received spleen cells identified by iso-antigens were given

950 R, a dose shown to be 100 per cent lethal to control mice (Barnes and Loutit, 1954c). Those mice which received immunologically activated spleen cells, and which were subsequently placed under the stress of repeated bleedings, were given a dose of 500 R.

Spleen cell transfer

Spleen cells to be identified by their iso-antigens were obtained from 8-day-old A mice. The spleens were mechanically minced by a rotating blade which reduced them to a suspension of individual cells and small cell clumps. The suspension was injected intravenously into irradiated and control CBA mice immediately after irradiation. Each host received $\frac{2}{5}$ of a spleen, approximately 15×10^6 cells.

Spleen cells to be identified by their production of antibody were obtained from adult donors. These spleens were also mechanically minced, and were injected into the peritoneum of irradiated and control mice at a dosage of one spleen per host.

Test for iso-antigens

Tissues to be tested for the presence of A iso-antigens were weighed, mechanically minced, and injected into the peritoneal cavity of CBA mice. Except in the case of peritoneal exudates, the tissues from each individual were tested separately, the quantities of tissue transferred being as follows: spleen, the whole organ; lymph nodes, the pooled axillary, brachial, inguinal nodes; liver, approximately 100 mg.; blood, 0·4 ml.; peritoneal exudate, containing 50–60 per cent macrophages and the rest lymphocytes, obtained from the peritoneal cavity 2 days after injection of 1 ml. paraffin (Mitchison, 1954).

After an interval of 8–12 days, the CBA hosts were inoculated subcutaneously with a standard test dose of 1×10^6 cells of the A strain sarcoma, Sarcoma 1. For the present study, the tumour was maintained in the ascites form by serial transfer in A mice. The tumours grown from the test inocula were excised and weighed after 8 days. Those mice which had been previously exposed to A iso-antigens, by the first injection of tissue, were expected to show smaller growths of tumour. Control groups of normal and well immunised CBA mice were included in each series inoculated with the tumour.

Transfer of antibody production

Spleen-cell donors were immunised by one or more intravenous injections of 0·2 ml. of a commercial preparation of *Salm. typhi* (H) antigen (Burroughs Wellcome standard agglutinable suspension). Separate samples of blood for agglutinin titration were obtained from each mouse by bleeding from the ventral tail artery.

RESULTS

Changes in spleen weight after irradiation

CBA mice which received a dose of 950 R of X-rays showed a rapid decline in the weight of the spleen. Without injection of spleen cells the irradiated mice died after $6{\cdot}3 \pm 0{\cdot}9$ days. Fig. 1 shows that injection of spleen cells brought about a gradual recovery in the weight of the host spleen, starting after 4–7 days. The possibility that the transplanted cells may have produced this recovery by colonising the host spleen is the subject of further investigation.

Sensitivity of the test for iso-antigens

Table I shows the growth after 8 days of the A tumour in untreated CBA mice, and in control CBA mice in which a state of immunity against A tissue had been previously induced. The control immunity was induced at least 15 days before the test inoculations of tumour, by subcutaneous inoculation of the same tumour, followed by growth and regression. Batches of normal and immunised mice were inoculated from the same pool of tumour cells, together with various experimental

groups of mice; each inoculum is numbered in the Tables, and some variation between inocula is apparent. The state of immunity is shown by the smaller growths of tumour among the mice which were previously inoculated. The mean weight of tumour, and its standard deviation, were calculated on a logarithmic scale for each batch of normal and immunised mice. Each mouse whose state of immunity was under test could then be assigned to either group.

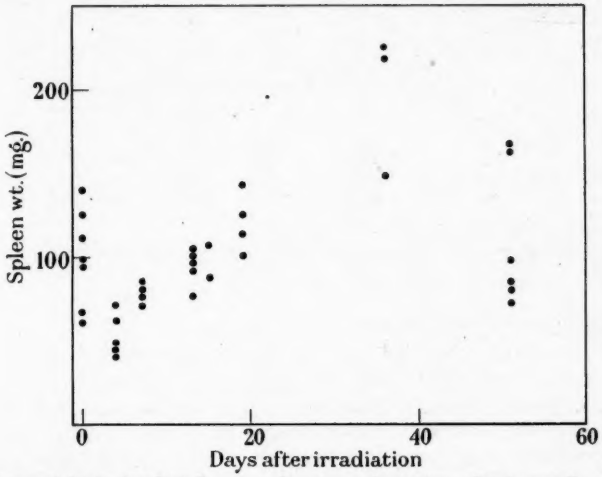

FIG. 1.—Spleen weight in irradiated mice after transplantation of spleen cells. Figs. 2–6 show titrations of individual sera, with points spaced separately for clarity. Each curve represents a single mouse.

The quantity of tissue from adult A mice needed to induce immunity is shown in Table II. A 1/125th fraction of a spleen (10^6 cells), or approximately one-tenth as many cells from a peritoneal exudate, is sufficient to produce a detectable state of immunity.

TABLE I.—*Growth of A Tumour in Normal and in Previously Inoculated CBA Mice*

Tumour inoculation no.	Weight (mg.) of tumour implants after 8 days.
	Normal CBA hosts.
1 . .	82, 146, 176, 212, 360
2 . .	104, 124, 128, 206, 218, 230
3 . .	48, 63, 66, 72, 126, 157, 197, 210
4 . .	332, 380, 442, 504, 637, 652
5 . .	192, 228, 232, 274, 294, 352, 450, 877
	CBA hosts previously inoculated with A tumour.
1 . .	8, 19, 25, 39, 42
2 . .	2, 2, 3, 4, 4, 9
3 . .	2, 2, 6, 10
4 . .	16, 17, 24
5 . .	7, 10, 12, 18

TABLE II.—*Estimation of the Quantity of A Tissue needed to induce Transplantation Immunity in CBA Mice*

(Weight (mg.) of tumour implants after 8 days.)

Tumour inoculation no.	Undiluted cell count.	Hosts previously injected with dilutions of cell suspension from one A spleen: 1/2.	1/5.	1/25.	1/125.	1/625.	1/3125.
1	151×10^6	—	8†	3†	3†	94*	—
3	120×10^6	3†	4†	2†	1†	—	—
5	178×10^6	—	12†	19†	22†	136	223
5	81×10^6	—	—	19†	96†	284	533
		Hosts previously injected with dilutions of A peritoneal exudate:					
3	$2{\cdot}8 \times 10^6$	2†	2†	27*	—	—	—
		9†	—	—	—	—	—
5	$4{\cdot}3 \times 10^6$	33*	9†	19†	90*	196	—
		65*	202	108*	352	772	—

* Significantly less than controls in normal hosts.

† Significantly less than controls in normal hosts, and not significantly more than controls in immunised hosts.

Fate of iso-antigens in irradiated and normal hosts

Table III shows the results of tests for the presence of A strain iso-antigens in tissues of the irradiated CBA mice which received spleen cells from A donors. Tests of various tissues obtained from single irradiated animals are shown on each line. No A strain iso-antigens were detected in liver or blood. Some variation between individuals is apparent in respect of the spleen, lymph nodes, and peritoneal exudates; but over the full duration of the investigation, from 4 until 51 days after irradiation, the iso-antigens of the transplanted cells could be detected in these sites in at least some animals.

The results of similar tests, carried out on the tissues of normal CBA mice which received spleen cells from A donors, are shown in Table IV. The iso-antigens of the transplanted cells were detected in the spleen after 4 and 7 days, but not later. No indications were found of their presence in the lymph nodes or blood.

Quantitative tests carried out on the spleens of irradiated mice did not give fully reliable results. But indications were given that a large fraction of the spleen may be of the donor antigenic type after irradiation and injection of spleen cells.

Antibody production in irradiated and normal mice receiving immunologically activated spleen cells

Preliminary experiments, shown in Fig. 2, established that *Salm. typhi* (H) antigen stimulated adequate production of antibody, and that irradiation is an effective agent in impairing production of this antibody.

Immunologically activated cells from the spleens of immunised mice were then transferred into irradiated and into normal host mice. In one series, shown in Fig. 3, the donors received a single injection of antigen 10 days before the transfer. In another, shown in Fig. 4, the transfer was carried out 4 days after a second injection of antigen into the donors. Both series show greater production of antibody in the irradiated than in the normal hosts. Donors and hosts were both

TABLE III.—*Detection of A Iso-antigens in Irradiated CBA Mice, After Transplantation of Spleen Cells.*

Tumour inoculation no.	Interval after irradiation (days).	Tissue transferred from irradiated mice; weight (mg.) of subsequent tumour implants after 8 days.				
		Spleen.	Lymph nodes.	Peritoneal exudate.	Blood.	Liver.
5	4	14†	388	—	—	—
		6†	42*	—	—	—
		—	116*	—	—	—
		—	62*	—	—	—
		—	40*	—	—	—
4	7	43*	198*	—	—	—
		219	104*	—	—	—
		135	268	—	—	—
		284	124*	—	—	—
		21†	270	—	—	—
5	13	—	30*	—	—	—
		—	16†	—	—	—
		—	144	—	—	—
		—	64*	—	—	—
		—	12†	—	—	—
1	14	38†	54†	—	277	72
		22†	168	—	275	135
		369	10†	—	93	105
		40†	60	—	94	306
		57†	17†	—	205	102
2	16	—	—	3†	—	—
		—	—	1†	—	—
2	19	40*	—	—	—	—
		21*	—	—	—	—
		4†	—	424	—	—
		61*	—	60*	—	—
		98	—	151	—	—
3	36	—	—	7†	—	—
		—	—	3†	—	—
4	51	32*	21†	—	—	—
		19†	22†	28†	—	—
		356	20†	—	—	—
		18†	12†	—	—	—
		68*	16†	38*	—	—
		47*	160	—	—	—

* Significantly less than controls in normal hosts.
† Significantly less than controls in normal hosts, and not significantly more than controls in immunised hosts.

of the A strain in the first series; in the second strain the donors were all of the A strain, so that a comparison could be made of homologous transfer with transfer into a foreign strain. The relationship of donor and host does not appear to affect the outcome of the transfer under these conditions.

Impaired production of antibody in a foreign host should nevertheless be demonstrable, if the transplanted cells were again transplanted from A donors into

TABLE IV.—*Detection of A Iso-antigens in Normal CBA Mice, After Transplantation of Spleen Cells.*

Tumour inoculation no.	Interval after irradiation (days).	Tissue transferred from irradiated mice; wt. (mg.) of subsequent tumour implants after 8 days.		
		Spleen.	Lymph nodes.	Blood.
4	4	130*	318	—
		358	330	—
		121*	409	—
		397	990	—
		122*	559	—
5	7	422	—	—
		51*	—	—
		652	—	—
		326	—	—
		588	—	—
1	14	194	—	269
		374	—	114
		112	—	329
		178	—	100
		164	—	166
5	53	1156	—	—
		798	—	—
		566	—	—
		172	—	—
		236	—	—

* Significantly less than controls in normal hosts.

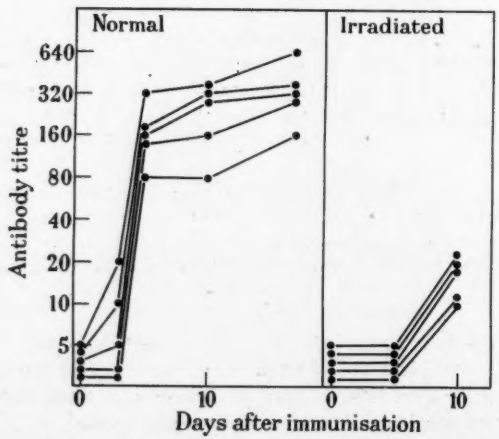

FIG. 2.—Antibody production in A mice after injection of *Salm. typhi* (H).

normal, and into irradiated CBA hosts, 4 days after a second injection of antigen. Half the normal, and half the irradiated hosts, had been previously immunised against A strain tissue by intravenous injection of 25×10^6 A spleen cells. Fig.

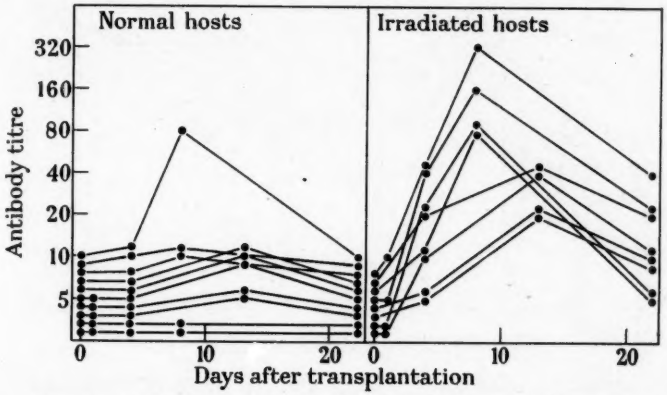

FIG. 3.—Antibody titres in hosts after transplantation of spleen cells from primarily immunised donors.

5 shows that production of antibody was impaired in the previously immunised hosts.

DISCUSSION

It has been shown that the iso-antigens of spleen cells which have been transplanted into irradiated mice are detectable in some of their hosts for at least 51 days, long after they have disappeared from normal hosts. This prolonged survival is evidence for the colonisation of the organs of the host by the transplanted cells; on this hypothesis, the host may properly be called a *radiation-chimaera*. An alternative hypothesis is that the iso-antigens of the transplanted cells become incorporated into the cells of the host. That this is the normal incorporation of foreign antigens into the tissues of the host is unlikely, in view of the large quantity of iso-antigen which was detected in some experiments, and the discrepancy between the normal and irradiated hosts. Cole *et al.* (1953) have discussed the possibility that material may be transferred to radiation-injured tissue analogously to bacterial transduction. This hypothesis is *prima facie* unlikely, but is not excluded by the present experiments.

It has also been shown that when spleen cells have been exposed to antigen and then transplanted, production of antibody is more active in hosts which have been previously irradiated. The present results with mice serve to confirm and extend the findings of Harris and Harris (1954) with rabbits. A source of confusion in the interpretation of this experiment is that antigen may be transferred together with the transferred spleen cells. But since irradiation has been shown to impair antibody production, immunological activation of host tissue cannot account for the discrepancy between normal and irradiated hosts. The enhanced production of antibody in the irradiated hosts may reasonably be explained as a result of multiplication of the transplanted cells.

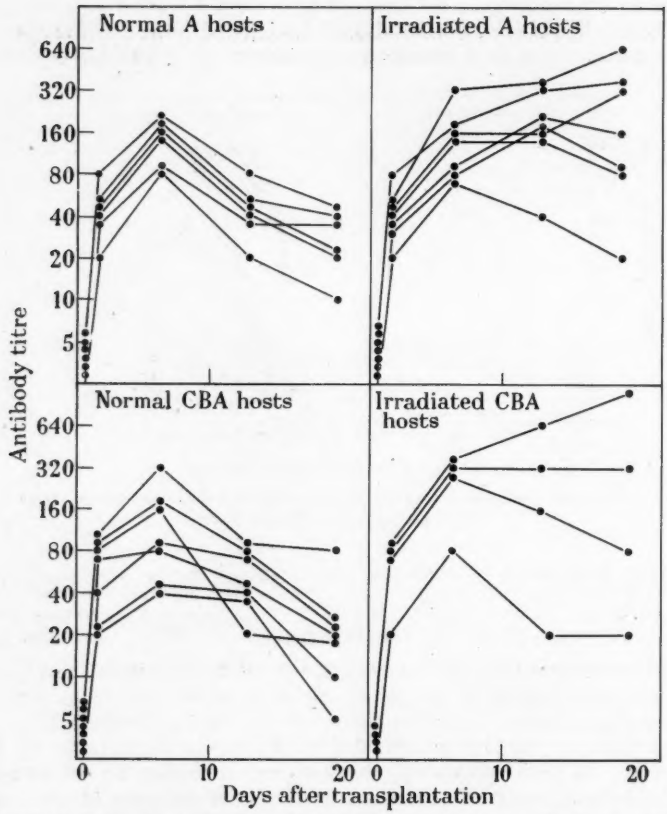

FIG. 4.—Antibody titres in hosts, after transplantation of spleen cells from secondarily immunised donors. Donors and hosts of the same strain (A) compared with donors and hosts of different strains (A and CBA).

The transferred iso-antigens were found only in the lymph nodes and spleen of the hosts. The transplanted cells therefore appear to have localised strictly in their homologous tissue. Similarly, Danchakoff (1918) and Ebert (1951) have shown that the embryonic spleen enlarges after grafting adult chicken spleen on the chorio-allantois.

SUMMARY

Spleen cells identified by iso-antigens were transplanted into irradiated mice. The iso-antigens were detected in the host lymph nodes and spleen up to 51 days after transplantation, by their ability to induce transplantation immunity.

Irradiation of the host enhanced the production of antibody by spleen cells transplanted from immunised donors.

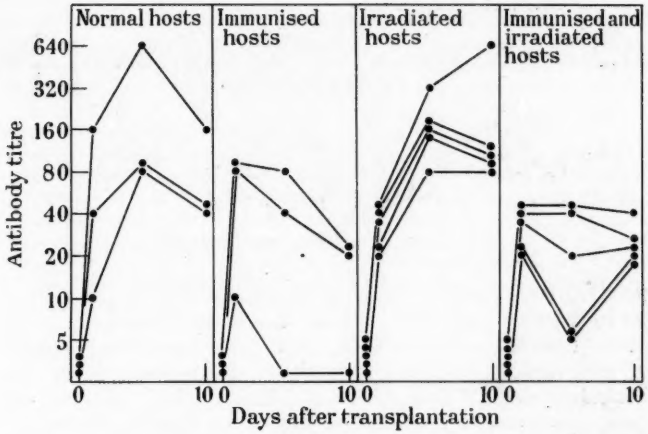

FIG. 5.—Antibody titres in hosts, after transplantation of spleen cells from secondarily immunised A donors. Normal CBA hosts compared with CBA hosts previously immunised against A tissue.

My thanks are due to the Director of the Atomic Energy Research Establishment for supporting this work, to Mr. M. Corp for carrying out the irradiation, and to Dr. D. E. H. Barnes and Dr. J. F. Loutit for advice, help and encouragement. Technical assistance was given by Miss Janet Hoare.

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EXPERIMENTALLY INDUCED OCULAR TOXOPLASMOSIS IN RABBITS

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EXPERIMENTAL ocular lesions due to a toxoplasma infection have been described by a number of workers (Levaditi, Schoen and Sanchis-Bayarri, 1928; Hogan, 1951; Busacca, Nobrega and Giovanni, 1952; Beverley, Beattie and Fry, 1954), but none of these lesions are similar to those that appear to be due to toxoplasma infections in man (Wilder, 1952*a*, *b*; Smith, 1954). The only lesions that are in any way comparable are those described by Frenkel (1955) in the golden hamster, and these lesions were not found in every animal.

Recently we have had the opportunity of studying a strain isolated from a fatal human infection in which the eyes showed the typical acute choroidoretinitis of toxoplasmosis. Since this strain was not lethal to rabbits, it seemed to be an ideal one with which to study the development of toxoplasma lesions in the eye.

MATERIALS AND METHODS

Toxoplasma strain

After isolation the strain was maintained by routine passage in mice. In the first few passages it was relatively poorly infective for mice, but after the twelfth passage there was an abrupt increase in virulence and from that time onwards mice died regularly 4–5 days after infection. (The strain was kindly provided by Prof. C. F. Barwell.) At the time of this work it had been propagated between thirty and forty times in mice.

Peritoneal fluid from an infected mouse, injected intradermally in rabbits at a dilution of 10^{-1} gave rise to a local necrotic lesion 1–2 cm. in diameter, which healed in approximately three weeks, leaving a hairless scar. During the first week of the infection the rabbit was febrile, but once the lesion ceased to spread the temperature subsided. One animal inoculated in this manner was found to be free of infection when examined post mortem six months later, having remained well in the intervening period.

Mice

Inbred mice used for transmission of the toxoplasma were of a Swiss albino strain in which no spontaneous toxoplasmosis has ever been found and in which serial passage of brain suspension had failed to unmask a latent infection. Mice were not tested for antibody before infection.

Rabbits

Rabbits used were purchased from dealers and were tested for toxoplasma complement-fixing antibodies before use. Only those in which antibody could not be demonstrated were used.

Toxoplasmin

Certain animals received toxoplasmin, and this was prepared from both egg- and chick-propagated toxoplasma by the method of Frenkel (1948). Standardisation was against a known human reactor.

Propagation of strain

Mice were infected by the intraperitoneal route receiving 0·2 ml. of undiluted peritoneal fluid. This caused death on the fifth day after infection. Peritoneal fluid for passage or infection was aspirated after killing the animals on the fourth day and all infection experiments were performed within 2 hr. of the fluid being removed.

Intraocular infection in rabbits

Animals were anaesthetised with nembutal and pantocaine was instilled into the eye over a period of 5 min. ; the superior rectus was held with fixation forceps and a needle was introduced into the vitreous at 11 o'clock, care being taken to avoid damage to the lens ; with the needle in the vitreous an injection of 0·05–0·1 ml. was made slowly so that there was no sudden increase in tension in the eye leading to oedema of the cornea.

All material injected in this manner must be sterile, as organisms normally non-pathogenic, such as *Bacillus subtilis* (Poplawska, 1891) and *Bacillus cereus* (Davenport and Smith, 1952), can cause panophthalmitis.

Complement fixation tests

Antigens were prepared by the technique of Sabin (1949), and were standardised against a known serum. All tests were performed using 2 M.H.D. of complement.

Histology

Eyes were removed immediately after death and placed at once in formol saline. Most eyes were sectioned after embedding whole in celloidin, but on occasions part of an eye was embedded in paraffin wax.

RESULTS

Clinical Course

When peritoneal exudate or a 10^{-1} dilution of the exudate from a mouse was injected into the vitreous there was such a gross reaction that the eyes were lost within 48 hr. To prevent this and to reduce reaction to the introduction of foreign protein (although it is unusual for there to be more than a transient reaction to a single intraocular injection of a foreign protein), the inoculum was diluted to 10^{-2} or 10^{-3}. A dilution of 10^{-3} contains approximately 5×10^2 lethal doses per ml. as titrated by intracerebral inoculation in mice.

During the first 24–48 hr. there was a minimal conjunctival injection, while the retina opposite and below the site of injection showed areas of oedema ; after a further 48 hr. the redness of the conjunctiva had passed off and apart from persistent retinal oedema the eye appeared normal. On the 8th–10th day after inoculation an acute iritis developed while by the 14th day a dense vitreous exudate was present.

About the third week the iritis and vitreous haze were clearing and localised white patches of choroiditis could be seen, while some of the retinal oedema still persisted. These patches gradually became less active and by the third month the appearances were those of a healed choroiditis.

The lesions were similar in animals receiving inocula of either 10^{-2} or 10^{-3}, although in the latter they were less severe.

Effect of Immunisation with Toxoplasmin on the Ocular Reaction

Five animals were given intravenous injections of toxoplasmin over a period of three months until they developed a complement-fixing titre of 64. This usually

required six injections of 2 ml. of toxoplasmin at intervals of a week with a booster dose fourteen days after the end of the course.

Animals given this treatment were infected intraocularly in the same way. Instead of a biphasic type of reaction with a quiescent period between the 4th and 11th days there was an intense iritis which began within 36 hr. reaching maximum severity in 3–4 days and in one instance becoming haemorrhagic. Symptoms of the acute phase gradually subsided and were clearing by the time that the second phase of reaction was seen in the non-immune animals.

Later lesions appeared similar to those seen in non-immune animals, but posterior synechiae were more frequently seen in this group.

It seemed possible that much of this severe iritis might have been due to a sensitivity to a foreign protein introduced into the vitreous of a sensitised animal as is seen in classical serum uveitis. In an attempt to reduce this effect, animals were immunised with both mouse- and egg-produced toxoplasmin and then challenged with toxoplasma grown in the opposite way, *i.e.*, egg toxoplasma-immunised animals received mouse toxoplasma and mouse toxoplasma-immunised animals were challenged with egg-grown parasites. In both cases the reactions were of the acute allergic type described above, but were rather more severe in those receiving the mouse infection, possibly because the mouse peritoneal fluid contained more viable toxoplasma than did a chick suspension. Unfortunately this experiment, although suggesting that the toxoplasma is responsible for the ocular allergic type of reaction, is not conclusive as it has been stated that there may be some cross reaction between chick and mouse proteins under these conditions (Beverley, 1955, personal communication).

Histology of the Retinal Lesions

Rabbits were sacrificed 2 weeks, 8 weeks and 5 months after infection. The retinal lesions did not appear to be modified by injection of toxoplasmin and appearances apply to both groups of animals.

Second week

There was a choroidal and retinal reaction which was most marked at the posterior pole of the eye below the medullated nerve fibres ; macroscopically this appeared as a white exudate lying on the retina. Microscopically the retina was becoming necrotic with a loss of normal pattern, while both it and the choroid were becoming filled with polymorphs, plasma cells and lymphocytes (Fig. 1), a picture similar to that seen in the infantile type of human choroido-retinitis. Eosinophils were also present in large numbers, but are not indicative of toxoplasmosis as they are frequently seen in bacterial infections of the rabbit eye. In the most severely affected areas free and proliferating toxoplasma could be seen (Fig. 2 and 3).

Eight weeks and five months

In general the appearances were the same in all eyes sacrificed after eight weeks. On opening the eye the most marked macroscopic feature was the choroido-retinal atrophy, and in one eye a retinal fold was found. Microscopically the retina had lost all normal structure and was thinned and atrophic. In areas of the atrophic retina, pseudo-cystic forms of the parasite could be found (Fig. 4).

Isolation of Toxoplasma from Infected Eyes

In order to confirm that the histological appearances were, in fact, due to toxoplasma and not to a non-specific reaction, attempts were made to re-isolate the parasite from the uveal lesions in rabbits sacrificed 2 and 8 weeks after infection. The uveal tissue was suspended in saline and the homogenate was injected intraperitoneally in mice; after twelve days the mice began to show symptoms of illness and by the 14th day were dying. Peritoneal smears showed the presence of typical toxoplasma.

DISCUSSION

Until the work of Wilder (1952*a*, *b*) the possibility of a primary ocular toxoplasma infection was largely ignored; earlier workers had described choroidoretinitis as one of the important features of congenital toxoplasma infection and isolations of the parasite had been made from the brain tissues of fatal human cases. Serological investigation was limited to the diagnosis of cases in children in whom it was assumed that infection had occurred *in utero*. This apparent neglect of the acquired form of the disease is even more surprising in view of the similarity between the retinal lesions of some of the congenital cases (Jankû, 1923; Woolf and Cowen, 1937), and that of many acquired cases of the disease.

Wilder's (1952*a*, *b*) observations were criticised by Sabin (1953) who pointed out that the bodies Wilder described could only be identified with certainty if they could be cultivated. Proof of the identity was provided by Jacobs, Fair and Bickerton (1954), who were able to obtain toxoplasma from a single excised eye in which choroidoretinitis was present.

A further method of proof of the toxoplasmic nature of retinal lesions would be by the experimental production of similar lesions. Attempts to do so date from Levaditi *et al.* (1928) who mention trying intraocular injection but do not adequately describe the lesions produced. Beverley *et al.* (1954) and Jacobs (1955, personal communication) infected rabbits intraocularly without producing retinal lesions; while the retinal lesions seen in the acute disease produced by Hogan (1951) by intracarotid inoculation of rabbits can hardly be compared with the human form of the disease. Thus this appears to be the first production of the human type of retinal lesion.

There is evidence which points to human infection being due to strains of low virulence, since up to 25 per cent of the population have serological evidence of infection without clinical disease. Similarly with ocular disease Woods, Jacobs, Woods and Cook (1954) and Smith and Ashton (1955) estimated that 20 per cent of cases of choroidoretinitis were probably due to toxoplasmosis, and in these patients the disease is a chronic one. It therefore appeared that any attempt to produce an experimental lesion exactly comparable to that found in the human eye would more likely be successful if the parasite employed had a low virulence for its experimental host. When the present strain was found it seemed to possess the required properties and, as this paper reports, it has been shown to give rise to lesions similar to those of the human disease. It seems that this success is probably due only to this difference in virulence, as in other respects the strain is similar to the classic strains of toxoplasma.

These results by providing an analogue of the human disease lend additional support to the view that the toxoplasma is an important aetiological agent in

human choroidoretinitis, and the chronic lesions described may provide more suitable lesions for testing chemotherapeutic compounds than the conventional tests on acute experimental infections hitherto employed.

SUMMARY

A recently isolated strain of toxoplasma has been shown to be able to produce retinal lesions similar to those seen in humans, when injected into the vitreous of rabbits. The strain is simple to work with in rabbits as it is non-lethal for these animals. The specific nature of the lesions has been confirmed histologically and by isolation of the toxoplasma from them.

Since this paper went to press I have been informed by Dr. L. Jacobs that since his previous communication to me referred to on page 251 he has succeeded in producing lesions similar to those described in this paper using the same technique.

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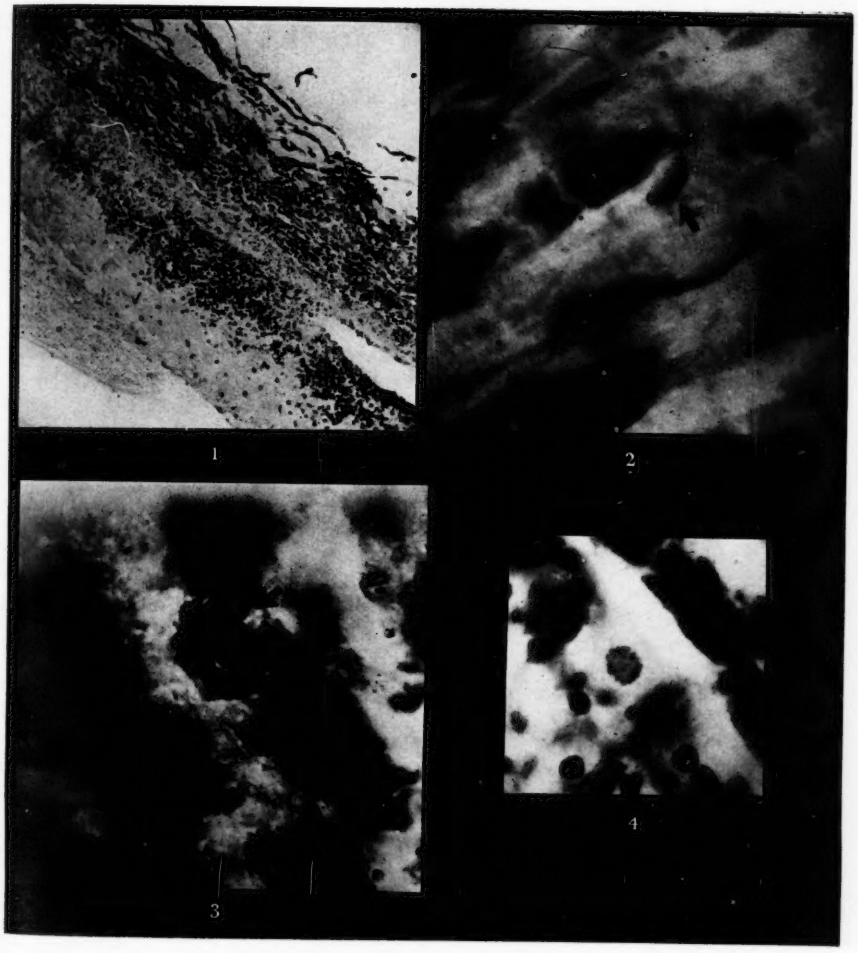
EXPLANATION OF PLATES

Fig. 1.—Rabbit choroidoretinitis, two weeks after an infection with toxoplasma, showing loss of retinal pattern and the retina and choroid filled with polymorphonuclear and mononuclear cells. × 120.

Fig. 2.—Single free crescentic toxoplasma in the rabbit retina. Two weeks after intraocular infection. × 1250.

Fig. 3.—Proliferating toxoplasma in necrotic choroid. Two weeks after intraocular infection. × 1250.

Fig. 4.—Pseudo-cystic form of toxoplasma in retina. Eight weeks after intraocular infection. × 900.



Smith.

THE *IN VITRO* PRODUCTION OF AMMONIUM AND AMINES BY INTESTINAL BACTERIA IN RELATION TO NITROGEN TOXICITY AS A FACTOR IN HEPATIC COMA

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LARGE amounts of ammonium salts are normally present in portal blood and are then metabolised to urea in the liver (Bollman and Mann, 1930). In patients with poor liver function and a portosystemic collateral circulation, and also in dogs with a portocaval shunt (Eck fistula), ammonium may by-pass the liver and reach high levels in the general circulation (Burchi, 1927; White, Phear, Summerskill and Sherlock, 1955). There is evidence that the presence of ammonium salts in the peripheral circulation may have toxic effects. For example, Bollman and Mann (1930) found that ammonium salts were toxic to dogs with an Eck fistula. A correlation between a high peripheral blood ammonium level and nervous symptoms has been noticed particularly after administration of a high protein diet in Eck fistula dogs (Monguio and Krause, 1934) or urea in man (Phillips, Schwartz, Gabuzda and Davidson, 1952) or after haemorrhage from intestinal varices (Riddell, 1955).

In addition to the possible toxicity of ammonium salts there is some evidence that methionine breakdown products, other than ammonium, may also be toxic. Thus, Phear, Ruebner, Sherlock and Summerskill (1956) have shown that after oral methionine certain cirrhotic patients exhibit nervous disturbances clinically indistinguishable from hepatic coma. In view of the fact that ammonium in particular is found in very high concentrations in the portal vein it seems probable that its source is the intestine (Cholopoff, 1927). Since bacteria are known to produce ammonium from both the breakdown of amino acids and the splitting of urea, it seems likely that the ammonium in the portal vein is the result of bacterial metabolism in the intestine. Attempts have therefore been made to treat cases of hepatic coma by the administration of antibacterial agents. Farquhar, Stokes, Whitlock, Bluemle and Gambescia (1950) found that chlortetracycline benefited such patients while Phear *et al.* (1956) found that the nervous disturbances following oral methionine, mentioned above, were partly or completely prevented by the administration of the same antibiotic. Dintzis and Hastings (1953) and Chao and Tarver (1953) found that the feeding of sulphonamides to animals inhibits the formation of ammonium from urea in the intestine. Sulphonamides have also been shown to lower the blood ammonium level in a patient with a porto-caval anastomosis (McDermott and Adams, 1954). Again, Martini, Phear, Ruebner and Sherlock (1956) during an investigation of the intestinal flora of cirrhotics found that when ileal fluid was incubated there was an increase of coliforms and *Streptococcus faecalis* with production of ammonium and break-

* In receipt of a grant from the Medical Research Council

down of methionine. However, the addition, before incubation, of chlortetracycline to the ileal fluid prevented almost completely the multiplication of the bacteria and the biochemical changes.

The object of the present study was to investigate the bacterial production of ammonium under *in vitro* conditions approximating to those probably existing in the intestine. The production of amines which may also be toxic (Melnykowycz and Johannson, 1955) was also examined. Work on bacterial nitrogen metabolism has mainly dealt with the action of bacterial suspensions on individual nitrogenous substances. We studied the changes produced by bacteria growing in media containing a mixture of amino acids. The strains used were intestinal bacteria isolated during the investigations of Phear *et al.* (1956) and Martini *et al.* (1956). An attempt was made to identify the principal ammonium producers and to study the effect of chlortetracycline on their growth and ammonium production with a view to applying rational chemotherapy to this condition or to changing the intestinal flora by dietary means.

MATERIAL AND METHODS

Strains of bacteria

The organisms used in this investigation were isolated mainly from the faeces of patients with cirrhosis of the liver before and after chlortetracycline therapy (Phear *et al.*, 1956). A few were obtained from the ileal fluid of cirrhotic patients (Martini *et al.*, 1956) and a few others from normal subjects. Strains representing each of the commoner intestinal organisms were chosen.

Sensitivity to chlortetracycline

This was measured by a tube dilution method in 0·5 per cent glucose broth (pH 7·2). The concentrations of chlortetracycline used varied from 250 to 0·12 μg./ml. Results were read after 18 hr. incubation for aerobes and after 2 days' incubation in a Fildes McIntosh jar for anaerobes. The end-point was taken as the lowest concentration of the antibiotic which inhibited growth. The Oxford Staphylococcus was used as a standard organism throughout. The results of sensitivity tests were in general agreement with those of other investigators (Ungar, 1951).

Culture media

A synthetic amino acid medium of known composition was used for most of the work. The amino acid mixture chosen was that of Stokes, Gunness, Dwyer and Caswell (1945). 'Yeastrel' was added to 0·5 per cent to supply the various members of the B group of vitamins. To each litre of medium were added 5 g. NaCl, 10 mg. $MgSO_4$, 7 g. Na_2HPO_4, 3 g. KH_2PO_4. The pH was adjusted to 7·2. The final nitrogen concentration was 130 mg. per cent. The amino acids remaining after incubation were identified by paper chromatography.

A casein hydrolysate medium was used for some of the work, and had the same composition except that the 130 mg. per cent nitrogen was derived from casein by acid hydrolysis.

A peptic digest of blood was prepared similar to that of Fildes (Mackie and McCartney, 1953) except that horse blood was substituted for sheep blood. Its nitrogen content was adjusted to 130 mg. per cent. Yeastrel and salts were added in the same concentrations as in the amino acid medium.

The buffer concentration (0·02 M) was insufficient to maintain the pH in the presence of added carbohydrate, and after incubation the pH fell. The final pH values of cultures inoculated with an atypical coliform (*Bacterium freundii*), *Proteus morgani* and a non-haemolytic streptococcus in the presence of glucose were respectively 5·2, 4·9 and 4·4.

Anaerobic conditions, when necessary, were obtained by boiling the medium and then adding 0·01 per cent thiolacetic acid. Other substances when added were present in the following concentrations: glucose 500 mg., lactose 500 mg. and urea 100 mg. per cent.

Medium in 10 ml. quantities was placed in 20 ml. screw-capped bottles, inoculated with a drop (0·02 ml.) of a broth culture of each strain and incubated at 37°.

Assessment of bacterial growth

After incubation, growth was usually assessed by the turbidity of the medium. An EEL photoelectric nephelometer (Evans Electroselenium Ltd.) was used. In order to obtain comparable readings the instrument was adjusted each day so that an opacity standard read 100 arbitrary units. The amount of bacterial growth of each species was assumed to be proportional to the nephelometer reading in arbitrary units. In order to compare the growth of different species it was decided to assess bacterial growth in μg. N/ml. One strain of each genus was grown in 0·5 per cent glucose broth, washed three times in 0·85 per cent sodium chloride and re-suspended. The N content of a suspension of standard turbidity (100 arbitrary units) was then determined.

Growth in the blood digest medium was not assessed turbidimetrically as turbidity estimations gave irregular results believed to be due to a breakdown product derived from the medium. When this medium was used in an experiment, growth was assessed by viable counts using the method of Miles and Misra (1938). It was realised that this was less reliable for the assessment of growth than a nephelometric method.

Time of incubation

To find the most suitable time for incubating the cultures before measuring growth and ammonium production a series of cultures of *Bact. freundii* was set up and their turbidity, viable count and ammonium content were measured daily. Growth and ammonium production were almost maximal after 1 day. Cultures of *Bact. freundii* and other aerobes were therefore examined after 18 hr. incubation. Anaerobes including microaerophilic streptococci and lactobacilli grew more slowly and were examined after 48 hr.

Biochemical methods

Ammonium production was measured by the microdiffusion method of Conway (1950). Total nitrogen was measured by Nesslerisation.

DL-methionine was measured by running the specimens, together with a range of standards, on buffered filter paper (McFarren, 1951) and then extracting and measuring the concentration of methionine in the spots by the method of Naftalin (1948) or of Yemm and Cocking (1955).

After making the medium alkaline, amines were extracted into ether. They were then re-extracted into a small volume of hydrochloric acid and run on unidimensional chromatograms using butanol-acetic acid as solvent. Chromatograms were run on Whatman No. 1 filter paper at room temperature. They were sprayed with ninhydrin. It was not possible to identify the amines fully, but known amines were run on each chromatogram, and their Rf values compared with those of the amines produced by the bacteria. No exact quantitative measurements of amine production were made, but the amount of substance in each spot was graded 0 to 6.

RESULTS

Growth and ammonium production by different species

The ammonium content of the medium was increased by the growth of all the species investigated except the lactobacilli which in some experiments did not produce any ammonium (Table I). In the absence of glucose the various species did not differ in the quantities of ammonium produced in relation to growth expressed in terms of

$$\frac{\text{production of } NH_4 \text{ nitrogen}}{\text{production of bacterial protein nitrogen.}}$$

The addition of glucose to the medium, while increasing the growth of all strains except *Bact. freundii*, decreased NH_4 production This agrees with the findings of Stephenson and Gale (1937). In the presence of glucose the most potent NH_4 producers were the coliform, *Proteus* and *Bacteroides* strains and the NH_4N produced in relation to growth was greater from Gram-negative bacteria

TABLE I.—*NH_4N Production by Different Species of Bacteria.*

Species.	Glucose. − Growth.	− NH_4N production.	− NH_4N/ growth.	Glucose. + Growth.	+ NH_4N production.	+ NH_4N/ growth.
Gram-negative bacteria						
Bact. freundii	109 (27)	163 (11)	1·5	68 (11)	109 (16)	1·6
Bact. coli	—	—	—	122 (38)	95 (12)	0·8
Bact. aerogenes	—	—	—	146 (39)	71 (21)	0·5
Bact. alkaligenes	29 (2)	71 (1)	2·4	—	—	—
Proteus mirabilis	36 (0)	184 (16)	5·2	127 (53)	133 (47)	1·1
P. vulgaris		—		153 (38)	82 (6)	0·5
P. morgani	78 (2)	150 (1)	1·9	149 (26)	128 (28)	0·9
Bacteroides	—	—	—	13 (23)	17 (6)	1·3
Ps. pyocyanea	—	—	—	155 (23)	83 (62)	0·5
		Mean	2·8		Mean	0·9
Gram-positive bacteria						
Cl. welchii	—	—	—	182 (91)	40 (6)	0·2
Cl. sporogenes	—	—	—	144 (77)	108 (74)	0·8
L. plantarum	—	—	—	27 (15)	4 (1)	0·2
L. bifidus	—	—	—	99 (65)	8 (15)	0·08
Staph. pyogenes	14 (2)	42 (11)	3·0	74 (56)	36 (18)	0·5
Str. faecalis (haemolytic)	—	—	—	79 (39)	25 (18)	0·3
Str. faecalis (non-haemolytic)	10 (3)	48 (1)	4·8	95 (19)	39 (1)	0·4
Microaerophilic streptococcus	—	—		21 (24)	14 (13)	0·6
		Mean	3·9		Mean	0·4

All cultures were grown in the amino acid medium of initial pH 7·2. Average growth and NH_4 production in μg.N/ml. Standard deviations in brackets.

(mean 0·9, range 0·5–1·6) than from the Gram-positive organisms (mean 0·4, range 0·08–0·8).

Altering the pH of the glucose-containing synthetic amino acid medium to pH 6 and pH 8 had little effect on the growth and ammonium production of *Bact. freundii*, *P. mirabilis*, *L. acidophilus* and *Str. faecalis*.

The substitution of lactose for glucose did not lower the NH_4 production of *Bact. freundii* to the same extent as glucose although this organism ferments both sugars. On the other hand, while *P. morgani* (a non-lactose fermenter) was unaffected by lactose, the growth and NH_4 production of *Str. faecalis* in this sugar did not differ from those in glucose.

Comparison of ammonium production in an amino acid, a casein hydrolysate and a blood digest medium

Ammonium production in relation to growth was similar in the amino acid and casein media but much less in the blood digest medium. The initial ammonium content of the casein medium was 400 μg. N per ml., while those of the amino acid and blood media were 43 and 62 μg. N per ml. Thus neither growth nor ammonium production was related to the initial ammonium content of the medium. In the blood medium there was little difference in the ammonium production in relation to growth by the three strains but in the casein and amino acid media *Bact. freundii* was the most potent ammonium producer followed by *P. mirabilis* and *Str. faecalis* (Table II).

TABLE II.—*Comparison of Growth and Ammonium Production in Amino Acid, Casein and Blood Media*

Species.	Amino acid. Viable count × 10^9/ml.	Amino acid. NH_4N production.	Amino acid. NH_4N/viable count.	Casein. Viable count × 10^9/ml.	Casein. NH_4N production.	Casein. NH_4N/viable count.	Blood. Viable count × 10^9/ml.	Blood. NH_4N production.	Blood. NH_4N/viable count.
Bact. freundii	1·6	142		2·5	126		2·9	29	
	2·5	139		3·0	135		2·5	30	
	2·0	102		2·9	155		4·7	41	
	1·9	119		3·0	151		—	—	
Average	2·0 (±0·3)	125 (±16)	63	2·8 (±0·2)	142 (±12)	50	3·4 (±1·0)	33 (±5)	9·7
Str. faecalis (non-haemolytic)	2·0	43		2·4	93		1·5	9	
	2·3	43		3·2	93		1·6	8	
	1·5	39		2·0	77		1·4	24	
	1·7	39		2·1	77		1·5	26	
Average	1·9 (±0·3)	41 (±2)	21	2·4 (±0·5)	85 (±8)	35	1·5 (±0·1)	17 (±8)	11
P. mirabilis	2·3	111		2·7	186		3·6	53	
	2·6	118		4·2	201		3·7	59	
	3·8	96		5·0	134		3·3	59	
	4·8	87		5·5	199		3·5	59	
Average	3·4 (±0·9)	103 (±13)	38	4·4 (±1·1)	180 (±27)	41	3·5 (±0·1)	57 (±8)	16

Glucose was added to all cultures.
Ammonium production expressed in μg. NH_4N/ml.

Urease activity

Table III shows the difference in ammonium production with and without added urea, among those strains found to possess urease activity. *Bact. aerogenes* and 3 species of *Proteus* were most active, producing more than 200 μg. per ml. more ammonium, in the presence of urea. *Staphylococcus pyogenes* and one strain of *Bact. coli* had less activity, producing about 20 μg. more ammonium, in the presence of urea. The breakdown of urea by *Bact. coli* confirmed the suggestion of Christensen (1946) that occasional strains might possess urease activity. The microaerophilic streptococcus and *Ps. pyocyanea* showed little activity, producing only about 5 μg. per ml. additional ammonium. No urease activity was observed in *Bact. alkaligenes*, a second *Bact. coli* strain, strains of *Str. faecalis*, lactobacilli, *Bacteroides* or *Clostridium welchii*.

The effect of chlortetracycline on growth and ammonium production

When carrying out chlortetracycline sensitivity tests it was found that tubes containing a concentration of drug which would just inhibit growth over 18 hr. showed good growth after 40 hr. This is probably accounted for by the instability of chlortetracycline (Ungar, 1951). Table IV shows the ammonium production in relation to growth in the presence of quantities of chlortetracycline which partially inhibited growth over a 40-hour period. It will be seen that chlortetracycline decreased the ammonium production in relation to growth of *Bact. aerogenes* and of a haemolytic *Str. faecalis* while having no effect on that of *Bact. coli*, *Bact. freundii*, *P. morgani*, *P. vulgaris*, *P. mirabilis* and *L. plantarum*. Ammonium production in relation to growth was increased in a micro-aerophilic streptococcus.

Amine production

A comparison was made of the amine production of *Bact. freundii*, *P. mirabilis* and a haemolytic *Str. faecalis* in the amino acid, blood and casein media, and in the last with and without added glucose. Under all conditions *P. mirabilis*

produced most amines, and casein was found to be the best medium for amine production by all species. The addition of thiolacetic acid slightly increased amine production. Glucose, while increasing the growth of all three species, decreased amine production by *Bact. freundii* and *Proteus*, while increasing that of the haemolytic *Str. faecalis*.

TABLE III.—*Urease Activity of Bacteria Possessing this Enzyme in an Amino Acid Medium Containing Glucose*

Growth and NH_4 production in μg.N/ml.

	Urea.				
	−		+		
Species.	Growth.	NH_4 produced.	Growth.	NH_4 produced.	Increase in the NH_4N production in the presence of urea.
Bact. aerogenes	183	81	183	468	387
			173	425	344
P. vulgaris	220	90	297	443	353
			258	417	327
P. morgani	189	166	239	500	334
			239	506	340
P. mirabilis	239	216	254	470	254
			254	423	207
Staph. pyogenes	151	53	138	98	45
			152	80	27
Bact. coli	178	101	156	123	22
			156	123	22
Anaerobic streptococcus	85	27	74	32	5
			83	31	4
Ps. pyocyanea	164	87	150	94	7
			155	89	2

TABLE IV.—*Effect of Chlortetracycline at Concentrations Just Limiting Growth on Ammonium Production in Amino Acid Medium Containing Glucose*

	Chlortetracycline									
	−					+				
	Growth.		NH_4N production.			Growth.		NH_4N production.			
Species.	Average.	S.D.	Average.	S.D.	NH_4N growth.	Average.	S.D.	Average.	S.D.	NH_4N growth.	Concentration of chlortetracycline (in μg./ml.).
Bact. freundii	104	(15)	123	(9)	1·2	86	(8)	96	(9)	1·1	2·5
Bact. coli	122	(40)	133	(22)	1·1	67	(4)	108	(17)	1·6	2·5
Bact. aerogenes	109	(24)	65	(24)	0·6	82	(20)	15	(9)	0·2	2·5
Str. faecalis (haemolytic)	68	(2)	45	(5)	0·6	55	(8)	10	(6)	0·2	50
P. mirabilis	141	(21)	107	(14)	0·8	109	(8)	103	(8)	0·9	50
P. morgani	117	(17)	155	(12)	1·3	94	(11)	173	(15)	1·8	50
P. vulgaris	125	(14)	122	(3)	1·0	77	(1)	147	(1)	2·0	50
L. plantarum	24	(5)	4	(4)	0·2	14	(4)	5	(4)	0·3	10
Microaerophilic streptococcus	38	(25)	23	(7)	0·6	10	(4)	25	(8)	2·5	10

It appeared that amines of Rf 0·04—0·06 are agmatine, histamine and cadaverine, while that of Rf 0·45–0·47 is probably tyramine with a front retarded by other substances present in the extract. It is not known to which amines the other substances correspond, but that of Rf 0·69–0·72 seems to be similar to the ephedrine-like spot of Melnykowycz and Johansson (1955). Spots with Rf of 0·21–0·23, 0·45–0·47 and 0·69–0·72 gave a positive Pauly's reaction.

Having determined that the casein medium was most favourable to amine production a comparison was made of amine production by these and some other species (Table V). Glucose was added to increase growth. *P. mirabilis* was most active, and lactobacillus, *Ps. pyocyanea*, *Bact. aerogenes* and *Bacteroides* less so. No amines were detectable in the medium after culture of *Cl. welchii* and *Bact. alkaligenes*. It was not possible to identify all the amines produced in our cultures but results are in general agreement with those of previous workers (Gale, 1946).

Chlortetracycline in the same subinhibitory concentrations as in Table IV prevented amine formation by *Bact. freundii* and *Bact. aerogenes* while having little effect on that of the haemolytic *Str. faecalis* and none on that of *P. mirabilis* (Table V).

TABLE V.—*Comparison of Amine Production by Different Species in a Casein Hydrolysate Medium with and without Subinhibitory Concentrations of Chlortetracycline*

Species.	Chlor-tetra-cycline.	Time of incubation in days.	Rf. 0·04–0·06	0·12–0·15	0·21–0·23	0·35–0·36	0·45–0·47	0·57–0·59	0·61–0·63	0·69–0·72	0·78–0·83
Bact. freundii	—	1	..	1	..	..	..	..	..	2	..
	+	1	—	..	..	..	..	..	..	..	..
P. mirabilis	—	1	1	1	..	2	2	4	3	5	1
	+	1	1	1	..	2	2	4	3	5	1
Str. faecalis (haemolytic)	—	1	..	..	..	..	3	..	2	3	..
	+	1	..	..	..	..	2	..	1	2	..
Cl. sporogenes	—	2	1	..	1	..	3	..	..	1	1
L. plantarum	—	2	..	..	..	..	..	..	..	1	..
Ps. pyocyanea	—	2	..	..	..	..	..	..	1	..	..
Bact. aerogenes	—	2	1	..	..	..	..	..	..	..	1
	+	2	—	..	..	..	..	..	..	..	..
Cl. welchii	—	2	—	..	..	..	..	..	..	..	..
Bacteroides	—	2	..	..	..	..	..	..	1	..	..
Bact. alkaligenes	—	2	—	..	..	..	..	..	..	..	..

All cultures were initially at pH 7·2 and were incubated anaerobically in the presence of glucose.
Numbers represent an estimation of the amount of ninhydrin-positive substance in each spot on an arbitary scale (0–6).

DISCUSSION

The results of our *in vitro* studies on ammonium production cannot unreservedly be applied to the more complex conditions existing in the intestine, although comparable quantities of ammonium were produced in incubation experiments using ileal fluid as a medium (Martini *et al.*, 1956). It would be of interest to know how much ammonium is produced in the gut daily. However, the volume of the gut fluid and its bacterial content are not known and are probably variable. The fact that in our cultures the bacterial cell nitrogen was of

the same order as the NH_4 nitrogen produced and that the nitrogen content of the faeces is 1–3 g. per day, suggests that this is in the order of g. rather than mg. White *et al.* (1955) showed that 3 g. of NH_4Cl may sometimes give rise to nervous symptoms in patients with cirrhosis of the liver. The quantity of ammonium produced by intestinal bacteria may therefore be of clinical significance. However, only work on isolated intestinal loops of animals can solve this problem satisfactorily. Ammonium production also occurred in a medium containing no other source of nitrogen than a peptic digest of blood. The rise in blood ammonium observed in cirrhotic patients after intestinal haemorrhage may therefore in part be due to ammonium produced by bacteria from blood in the intestine. When blood urea levels rise in cirrhosis of the liver the urease activity of *Proteus* and *Bact. aerogenes* strains may be of clinical significance.

In our experiments cultures were made in small screw-capped bottles in order to prevent escape of ammonium, and anaerobic conditions almost certainly prevailed during most of the incubation period. This assumption is supported by two observations. Firstly, *Bact. freundii* broke down aspartic acid, cysteine, threonine and serine, which it attacks by dehydrogenation, rather than glycine, alanine and glutamic acid which it would have attacked by oxidation (Fry, 1955). Secondly, the rate of ammonium production by *Bact. coli* is greatest under aerobic conditions (Stumpf and Green, 1944). Since we found no difference between the ammonium production of *Bact. coli* with and without thiolacetic acid, anaerobic conditions seem to have prevailed even without this reducing substance. As conditions in the large intestine are favourable to the growth of anaerobic bacteria we did not consider that our results were invalidated by anaerobiosis in our cultures. It must be admitted that in the intestine, where mixed bacterial populations compete for available nutrients, bacteria may not show the same degree of ammonium production as did our pure strains in relatively simple media. Although we found that in several species ammonium production at pH 6 and pH 8 did not differ greatly from that at pH 7, further work on ammonium production under more varied conditions is clearly required. In particular the metabolism of mixed cultures of intestinal bacteria and the production of ammonium during the early phases of growth seem to merit further investigation.

The intestinal flora of patients with cirrhosis of the liver differs from that of normal subjects in a greatly increased number of coliforms and *Str. faecalis* in the small intestine. These organisms also occur at a higher level in cirrhotics (Martini *et al.*, 1956). It does not seem probable, however, that the increased blood ammonium levels in these patients are due to this increase in small intestinal flora. Patients suffering from other diseases but with normal liver function may have a similar intestinal flora without showing any clinical or biochemical abnormality (Martini *et al.*, 1956). It seems probable, moreover, that even in patients with cirrhosis of the liver the majority of intestinal bacteria are situated in the large intestine, which therefore remains the main site of ammonium production. The increased blood ammonium levels in cirrhosis are therefore almost certainly not due to increased ammonium production in the intestine but to a decreased ability of the liver to metabolise ammonium or to the presence of a collateral circulation which by-passes the liver.

Reduction in ammonium production by the intestinal flora in patients with hepatic coma might be of benefit. As glucose was found to reduce ammonium production by intestinal bacteria the low protein–high carbohydrate diet

usually prescribed for cirrhotic patients may lower ammonium production partly by lowering the amount of nitrogenous substrate available to bacteria and partly because of the effect of glucose in lowering ammonium production. Lactobacilli which produce little or no ammonium are generally believed to become prominent in the intestinal flora when a milk diet is given (Dudgeon, 1926). Such a diet might therefore be of benefit to patients suffering from the nervous complications of liver disease.

Chlortetracycline was considered to be beneficial in hepatic coma by Farquhar *et al.* (1950) and more recently antibiotics have been advised for the treatment of hepatic coma on the supposition that production of harmful toxic metabolites might be diminished (Riddell, 1955). Investigations of the effect of chlortetracycline on the faecal flora (Phear *et al.*, 1956) showed that the total viable counts were hardly diminished. Although there was a constant depression of *Bacteroides* with a partial lowering of the coliform count, the number of *Proteus*, anaerobic streptococci and lactobacilli increased in many cases and *Str. faecalis* was unaffected. Replacement of *Bacteroides* and some coliform strains by *Proteus*, another strong ammonium producer, may have occurred in the Eck fistula dogs of Mann, Bollman, Huizenga, Farrar and Grindlay (1954) who found that oxytetracycline did not lower the blood ammonium levels in these animals. Our finding that chlortetracycline at subinhibitory levels may lower ammonium production *in vitro* is of interest but of limited importance as the proportion of strains showing this phenomenon was small.

Antibiotics affecting the principal ammonium producers, *Proteus*, coliforms and *Bacteroides*, should cut down the production of ammonium by intestinal bacteria and therefore might benefit patients in impending hepatic coma. Chlortetracycline only fulfils these conditions partially and other wide spectrum antibiotics such as neomycin (Poth, 1954) or a combination of two drugs would probably be more effective.

SUMMARY

A comparison has been made of the *in vitro* ammonium and amine production by different species of growing intestinal bacteria.

When cultivated in a synthetic amino acid medium containing glucose of pH 7·2 all species, except lactobacilli, produced ammonium. Gram-negative bacteria produced more ammonium than Gram-positive organisms. Glucose increased growth in almost all instances and decreased ammonium production. Ammonium production was greater in a casein hydrolysate and less in a blood digest medium than in the synthetic amino acid medium. Urease activity was high in *Proteus* and *Bact. aerogenes* and less in *Staph. pyogenes*, *Bact. coli*, *Ps. pyocyanea* and an anaerobic streptococcus.

Amine production was greater in the casein medium than in that containing synthetic amino acids or a peptic digest of blood. *Proteus* was the most active amine producer.

Chlortetracycline at subinhibitory levels in two strains reduced ammonium production out of proportion to its effect on growth.

As ammonium salts are toxic to patients with cirrhosis of the liver a reduction of intestinal ammonium production might be beneficial. The use of dietary measures and of antibiotics for this purpose is discussed.

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THE CHEMICAL BASIS OF THE VIRULENCE OF *BACILLUS ANTHRACIS* VII. TWO COMPONENTS OF THE ANTHRAX TOXIN: THEIR RELATIONSHIP TO KNOWN IMMUNISING AGGRESSINS

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SMITH, Keppie and Stanley (1955) demonstrated that *Bacillus anthracis* growing *in vivo* forms a specific oedema-producing toxin which is responsible for death of the host. This toxin, present in the plasma of guinea-pigs dying of anthrax, has not been produced by culture *in vitro*. It is unrelated to the capsule of the organism but closely connected with two preparations from *B. anthracis* which actively immunised animals against infection. These purified preparations were extracellular products of the organism, one isolated from an *in vitro* culture (Strange, and Belton, 1954) and the other separated from exudates of infected guinea-pigs. The latter was described in a previous paper (Smith and Gallop, 1956) and was called Fraction Y. Both preparations, when injected into a suitable host, produced antisera which neutralised the effects of the anthrax toxin, but possessed no toxic or oedema-producing activity. Both were antiphagocytic but had no virulence-enhancing activity. They were different in their chemical properties and in their behaviour in the ultra-centrifuge.

In the previous work (Smith and Gallop, 1956), the immunising aggressin Fraction Y was effectively separated by preparative ultracentrifugation after preliminary fractionation of plasma/exudate with salts and ethanol. This preliminary fractionation destroyed the already small toxic, oedema-producing and virulence-enhancing activities of the original plasma/exudate and consequently Fraction Y was devoid of such activities. When attention turned to toxic plasma which contained a much higher level of toxin than the plasma/exudate, the detrimental effect of such preliminary fractionation was known (Smith *et al.*, 1955) and was avoided. Direct ultracentrifugation of the toxic plasma led to the demonstration of two components of the anthrax toxin and to the recognition of an interesting relationship between them and Fraction Y and the immunising aggressin prepared *in vitro*. This work is described here together with a brief account of unsuccessful attempts to produce the anthrax toxin *in vitro*, which emphasise the importance of the *in vivo* environment in relation to toxin formation.

MATERIALS AND METHODS

Toxic plasma—was collected from guinea-pigs dying of anthrax, filtered sterile and freeze-dried as described by Smith *et al.* (1955).

Skin test for oedema-producing activity—as described by Smith *et al.* (1955). In some tests, the sizes of the lesions were noted and are reported here in the nomenclature used by Smith *et al.* (1955). In later experiments, biological assay of fractionation samples was carried out

by injecting serial descending dilutions of the materials under test and noting the null point, *i.e.*, the first dilution not giving a positive reaction.

Test for lethality to mice—as described by Smith *et al.* (1955).

Assay for immunising antiphagocytic and virulence-enhancing activites—as described by Smith and Gallop (1956).

Immunising aggressin produced in vitro—our colleague R. E. Strange supplied us with adequate quantities of a purified preparation. In this paper it is described as the *in vitro* antigen.

Immunising aggressin isolated from in vivo *sources*—this was Fraction Y from plasma/exudate (Smith and Gallop, 1956).

Ultracentrifugation—was carried out in the preparative angle rotor of the Spinco ultracentrifuge at 100,000 × g (40,000 r.p.m.) and at 0°–5°. In the text only the duration of ultracentrifugation is quoted.

Anthrax antiserum—hyperimmune serum prepared in the horse by injection of spores of the " Sterne " uncapsulated strain of *B. anthracis*.

EXPERIMENTAL AND RESULTS

Ultracentrifugation of Toxic Plasma

In the fractionation of plasma/exudate Fraction Y was deposited from a 1·0–1·5 per cent solution of protein (Fraction A described by Smith and Gallop (1956)) in 0·2μ phosphate buffer (pH 8) by ultracentrifuging for 4–6 hr. When the relatively viscous toxic plasma (protein content approx. 6 per cent) was treated similarly the time of ultracentrifugation had to be increased to remove the bulk of the toxic activity from the supernatant.

Freeze-dried toxic plasma was re-constituted to its original volume (15 ml.) with ice-cold water. Some insoluble material was removed by centrifugation

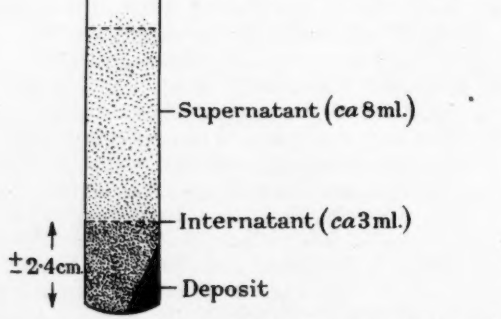

FIG. 1.—Diagram of the contents of a tube after ultracentrifuging the toxic plasma for 16 hr.

(10,000 r.p.m., 20 min.) at 0°, suspended in saline (3 ml.) and reserved. The supernatant (13–14 ml., sufficient to fill a tube of the rotor) was ultracentrifuged for 16 hr. When the tube was removed from the rotor the appearance of its contents was as shown diagrammatically in Fig. 1. *Clear Supernatant* and *Supernatant* were removed carefully with Pasteur pipettes into separate bottles for biological testing. Ice-cold saline (pH 7·4) was added to fill the tube which was then inverted

several times to disperse the internatant in the saline but not to dissolve the deposit; the material was then ultracentrifuged for 16 hr. After this second ultracentrifugation, the whole of the liquid was removed carefully from the deposit and concentrated on collodion ultrafilters to approx. 3 ml.; this constituted the *Internatant.* The deposit was dissolved in a quantity of ice-cold saline (pH 7·4; sufficient to fill the tube) and then ultracentrifuged for 6 hr. The supernatant was concentrated on collodion ultrafilters to approx. 1·5 ml. and constituted the *Washings.* The *Washed Deposit* was dissolved in ice-cold saline (1·5 ml., pH 7·4), centrifuged to remove a little insoluble matter, and reserved for biological testing. Table I summarises the yields and the results of tests for toxicity in 4–6

TABLE I.—*Toxicity Tests on Ultracentrifugal Fractions of Toxic Plasma*

Fraction.	Vol.* (ml.).	N (approx.) per cent.	Skin test (size of lesion) (ml.). 0·4.	0·2.	0·1.	0·05.	Mouse lethality test (ml.). 2.	1.	0·5.	0·25.
Soluble, re-constituted toxic plasma	13–14	1·1	—	8 : 38	7 : 32	6 : 30	—	10/10	—	—
Residue insoluble in water	3	1·4	—	3 : 18	*tr.*	—	—	0/2†	—	—
Clear supernatant	3–4	0·16	—	4 : 16	*tr.*	—	—	0/10	0/10	—
Supernatant (II)	7–8	0·7	6 : 30	5 : 22	*tr.*	—	6/25	1/25	0/25	—
Internatant	3	1·7	—	5 : 30	4 : 24	—	—	1/20	—	—
Washings	1·5	0·6	—	6 : 30	5 : 26	—	—	0/5	—	—
Washed deposit (I)	1·5	0·6	—	7 : 30	6 : 28	5 : 24	—	0/5	0/5	0/5
In vitro antigen	—	0·015‡	—	*Nil*	*Nil*	*Nil*	1/10	0/10§	0/10	—

* The yields from a single ultracentrifuge tube are quoted here. † Injected intraperitoneally. ‡ Equivalent to 0·1 per cent w/v solution. § Unaltered by dissolving the *in vitro* antigen in normal guinea-pig plasma.

similar experiments. The toxicity of the original toxin was largely lost in the fractions from it. Even the most highly active fraction, *i.e.*, the *Washed Deposit* was less active than the original toxin although eight times more concentrated than in the toxic plasma from which it was obtained.

A Synergic Mixture of the Deposit (Factor I) and Supernatant (Factor II) from the Toxic Plasma

Skin test and mouse lethality tests were carried out on mixtures of the *Supernatant* and the solution of the *Washed Deposit*, the amounts being equal to the smallest of those referred to in Table I. The average results of a number of similar experiments on these mixtures are shown in the first line of Table II. The toxicity of the mixture in skin and mouse lethality tests was greater than that shown separately by each component at four times their strength in the mixture (see Table I). *Washed Deposit* and *Supernatant* therefore contained two distinct components (Factors I and II respectively) of the anthrax toxin which formed a synergic mixture.

A number of materials were tested for ability to replace Factor II in the above tests. In skin tests, the lesion (5 : 24) produced by injection of the solution (0·05 ml.) of Factor I was not significantly increased by admixture with 0·1 ml. of sodium polyglutamate (0·5 per cent), normal guinea-pig plasma and its ultrafiltrate,

TABLE II.—*Synergic Mixtures of Washed Deposit (Factor I) with Supernatant (Factor II) and of Each Factor with the* in vitro *Antigen*

		Skin tests.				Mouse lethality tests.		
Mixture.†	Vol. (ml.).	Alone.	With* normal serum.	With* anti-serum.	Vol. (ml.).	Alone.	With* normal serum.	With* anti-serum.
Washed Deposit (Factor I)	0·05				0·25			
+		7 : 34	7 : 35	*Nil*		5/5	—	—
Supernatant (Factor II)	0·1				0·5			
Washed Deposit (Factor I)	0·05				0·25			
+		7 : 33	7 : 35	*Nil*		5/5	9/9	0/9
In vitro antigen (0·1 per cent)	0·1				0·5			
Supernatant (Factor II)	0·1				0·5, 1·0‡			
+		5 : 22	5 : 28	*Nil*		2/5, 8/10	5/5, 5/5	0/5, 0/10
In vitro antigen (0·1 per cent)	0·1				0·5, 1·0‡			

* 1/5 of the volume of the mixture. † Details of the preparation and strength of the components and their toxicity when injected alone see text and Table I. ‡ A 0·1 per cent solution of *in vitro* antigen in Supernatant—1 ml. injected.

ultrafiltrate from Factor II, a solution of $NaHCO_3$ (0·1, 0·02 and 0·004 per cent), "ballotini" and ammonium carbonate extracts of *B. anthracis* grown *in vivo* (Smith, Keppie and Stanley, 1953*a*, *b*), filtrates of cultures of *B. anthracis* (strain N.P.) in normal guinea-pig plasma or whole blood, A.T.P. (0·1 per cent) and 0·02 M solutions of $FeCl_3$, $FeSO_4$, $MgSO_4$, $CaCl_2$ and KCl. In mouse lethality tests no animals died when batches of 4 were injected with mixtures of a solution (0·25 ml.) of Factor I with 0·5 ml. of sodium polyglutamate (0·5 per cent) in saline, normal guinea-pig plasma and the ultrafiltrate from Factor II.

In view of the synergic action between the two factors the remaining fractions from ultracentrifugation were tested for the presence of Factors I and II by combining them in skin tests with the solutions of Factors II and I respectively. The residue insoluble in water and *Clear Supernatant* contained no significant amounts of either factor. As would be expected, the *Internatant* and *Washings* were mixtures of Factors I and II, the proportions of which varied somewhat from batch to batch. They were mixed together and treated by a similar process to that used on the original toxin to recover more of Factors I and II.

Synergic Mixtures of the In Vitro *Antigen with Factors I and II*

The solution of Factor I was mixed with the *in vitro* antigen, the amounts being equal to the smallest referred to in Table I. The average results of a number of similar skin tests and mouse lethality tests on these mixtures are shown in the second line of Table II. As with the mixture of Factors I and II, the toxicity of the mixture in both tests was greater than that shown separately by each component of the mixture at four times their combined strengths. The toxic action of the synergic mixture was specifically inhibited by anthrax antiserum.

Although the *in vitro* antigen will replace Factor II in skin and mouse lethality tests, the two materials are not identical. This was first shown by the fact that mixtures of the *in vitro* antigen with Factor II acted synergically and not additively in mouse lethality tests. Thus, in amounts equal to the two lowest of the three referred to in Table I, they formed mixtures significantly more lethal to mice (line 3, Table II) than the top concentration of each component (see Table I) when they were injected separately. The lethal action of the mixture was specifically inhibited by antiserum. These mixtures of Factor II with the *in vitro* antigen did not show a synergic action in the skin test.

Synergism of Mixtures of Factors I and II in Null Point Skin Assays; Inactivity of the In Vitro *Antigen*

For the further fractionation of Factors I and II a null point assay in the skin of guinea-pigs (see Methods) has been used. The following null points (average from 5–6 similar experiments) were obtained when the initial materials (0·2 ml.) were injected separately: original toxic plasma 1/128; solution of Factor I 1/64; and solution of Factor II (separated from the *Internatant* at a level a few mm. higher than in the previous experiments) 1/2.

Factor I (0·1 ml. of a 1/64 dilution) was mixed with Factor II (0·1 ml. of a 1/2 dilution). When injected, the mixture produced a positive skin reaction and could be diluted 1/8 before the null point was reached. If Factor II was replaced by the *in vitro* antigen (0·1 per cent) no skin reaction was produced. Null point assays were carried out in which one factor in the mixture of the two was held constant at half the strength of its null point when injected separately, while the other factor was serially diluted. In these, as in the first experiments, the mixtures of Factors I and II produced a synergic effect but mixtures with the *in vitro* antigen did not. Thus, a 1/64 dilution of Factor I (0·1 ml.) produced no skin reaction when injected alone or with a 0·1 per cent solution of the *in vitro* antigen (0·1 ml.) but on admixture with a solution of Factor II (0·1 ml.) the latter had to be diluted 1/16 before a skin reaction was not evoked. Similarly the solution of Factor II (0·1 ml.) produced no skin reaction when injected alone or with a 0·1 per cent solution of the *in vitro* antigen (0·1 ml.) but on admixture with a solution of Factor I (0·1 ml.) the latter had to be diluted 1/1024 before the null point was reached.

There does not seem to be a large excess of Factor I or II in the original toxin since addition of solutions of either Factor I (0·1 ml. of 1/64) or Factor II (0·1 ml. of 1/2) to the toxin (0·1 ml. of 1/128) formed a mixture which failed to produce a skin reaction on injection.

Stability of Factors I and II

Factors I and II retained both their inherent activity and that shown on recombination with a fixed and separately unreactive amount of the other, after freeze-drying and for at least 28 days in the dry state (*cf.* original toxin, Smith *et al.*, 1955).

In solution at 0°, both factors gradually lost activity especially if the solutions contained any slight bacterial contamination. For this reason, the solutions were filtered through "Millipore Filters" as soon as they were collected and freeze dried unless they were to be used immediately.

Further Purification of Factor I : Its Immunising and Aggressin Activity

The *Washed Deposit* (*ca.* 60 mg.) obtained from one ultracentrifuge tube of toxic plasma (13–14 ml.) was dissolved in ice-cold phosphate buffer (pH 8, 0·2 μ, 13–14 ml.). The solution was ultracentrifuged for 6 hr. and the process repeated 4 times. The final deposit (*ca.* 6 mg.) was dissolved in phosphate buffer (1 ml.) for biological testing. Analytical ultracentrifuge diagrams of the final deposit are shown in Fig. 2. The main component (S = approx. 15) in this diagram corresponds

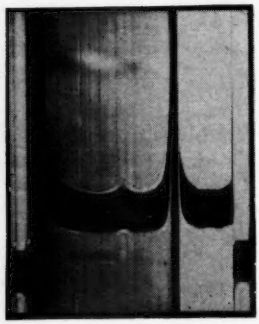

FIG. 2.—Analytical ultracentrifuge diagram of purified Factor I of the anthrax toxin. The conditions were the same as those described in Fig. 2 of Smith and Gallop (1956); the protein concentration was 1·2 per cent.

with Fraction Y (see Fig. 2(*e*) of Smith and Gallop, 1956); the small fast-moving component is present in normal guinea-pig plasma.

The toxic, immunising, and aggressin activity of the original *Washed Deposit* and the final deposit after 5 washings with phosphate buffer are given in Table III, which also includes a description of the behaviour of the Fraction Y in the biological tests. The final deposit retained an ability to form a synergic mixture with Factor II. Furthermore Factor I in this deposit was free from much impurity. Its anti-phagocytic and immunising activities were similar to those of Fraction Y, but in

TABLE III.—*Purification of Factor I and a Comparison with Fraction Y.*

Fraction.	Toxic action. Conc. (per cent protein).	Skin reaction (null point) dilution. Inherent activity.	Skin reaction (null point) dilution. Combined with† Factor II.	Mouse lethality test.‡ No. dead / No. injected.	Immunising* activity (lowest conc. giving 50 per cent protection).	Anti-phagocytic* activity (lowest active conc. per cent).	Virulence* enhancing activity (lowest active conc. per cent)
Factor I							
(*a*) Original Washed Deposit	4	1/64	1/1024	5/5	0·020 (0·007–0·058)	0·15	0·5
(*b*) Deposit—after washing five times in the ultra-centrifuge	0·6	1/32	1/512	7/9	0·004 (0·0013–0·012)	0·03	0·08
Fraction Y	0·5	Undiluted	Undiluted	0/9	0·006 (0·003–0·012)	0·02	Inactive at 0·5

* For details of these tests and methods of expressing activities see Smith and Gallop (1956). † Factor II held constant at 0·1 ml. of a 1/2 dilution, *i.e.*, half the strength of its null point. ‡ Solution (0·5 ml.) mixed with solution of Factor II (0·5 ml.).

contrast to the latter, which had no ability to form a toxic mixture with Factor II, it had marked virulence-enhancing properties. Further tests showed that this purified Factor I formed a toxic mixture with the *in vitro* antigen but Fraction Y did not.

The supernatant from the ultracentrifuging of the toxic plasma retained an immunising activity but a detailed investigation of the immunising and aggressin activity of Factor II cannot be made until some purification of it has been attained.

Attempts to Produce the Anthrax Toxin In Vitro

The following experiments were carried out in an attempt to produce the anthrax toxin *in vitro*. In contrast to the attempts of previous workers (Eurich and Hewlett, 1930 ; Sobernheim, 1931 ; King and Stein, 1950) we were aided by the knowledge that a small sublethal concentration of the toxin could be detected by a skin reaction ; it had been established that oedema-producing activity in the skin of a guinea-pig and mouse lethality were due to the same toxin (Smith *et al.*, 1955). Filtrates of numerous cultures were examined for toxin using this skin test. None of the preparations which are described below contained even the small amount of toxin detectable by this test. The cultures described represent a progressive approach towards the conditions occurring *in vivo*.

Growth in laboratory media

B. anthracis (strain N.P.) was grown at 37° for 16 hr. in tryptic meat broth and in the medium used by Belton and Strange (1954) to produce the *in vitro* antigen. No toxin was produced.

Growth in guinea-pig plasma

Cultures of *B. anthracis* in heparinised (10 i.u. per ml.) guinea-pig plasma were grown for 24 hr. under the following conditions : static, 37°, aerobic ; static, 37°, anaerobic ; static, 20°, aerobic ; static, 20°, anaerobic ; shaken, 37°, air containing 5 per cent CO_2 ; all were without activity. Since the plasma A.T.P., glutamine and $NaHCO_3$ levels were reduced by the growth of *B. anthracis* during the final bacteraemia of anthrax in the guinea-pig, A.T.P. (0·1 per cent), glutamine (0·1 per cent) and $NaHCO_3$ (1 per cent) were added separately to static 24 hr. cultures of *B. anthracis* in guinea-pig plasma but failed to stimulate toxin synthesis. Seven-hour cultures (37°) of *B. anthracis* in guinea-pig plasma alone and with the addition of yeast ribonucleic acid (0·1 per cent) or its alkaline digest (0·1 per cent) did not contain toxin.

To test whether related strains of organisms could produce the anthrax toxin some of the experiments described above were carried out using *B. anthracis* (Sterne), *B. anthracis* (Stamatin and Stamatin, 1936) and *Bacillus cereus*. Only cultures of the latter produced a skin reaction which was, however, not inhibited by anthrax antiserum.

Growth in infected blood

The blood of a guinea-pig infected with *B. anthracis* and approximately 8–10 hr. from death was removed by heart puncture. The bacterial count in the blood was approx. 1×10^6 chains per ml. (see Keppie, Smith and Harris-Smith, 1955) and only a small amount of toxin was detectable in the plasma. If the blood had not been removed until the animal died, the bacterial count would have been approx. $0{\cdot}5–1 \times 10^9$ chains per ml. (Keppie *et al.*, 1955) and approximately 30 × more anthrax toxin would have accumulated. The infected blood, or the plasma after removal of the red cells by slow centrifugation, was shaken in a Warburg apparatus for 8–10 hr. with continuous gassing using air containing 5 per cent CO_2. Growth of the bacteria occurred almost at the same rate as the growth *in vivo* but no significant amounts of toxin were produced. Addition of $NaHCO_3$ (0·1 per cent), of tissue slices (spleen, kidney or liver), of A.T.P. (1, 5 and 25 mg. per 100 ml.) and glutamine (0·1 per cent) to this system were without effect in producing toxin. Neither increasing the rate of shaking

and surface area of the liquid in the Warburg flask nor the use of static culture resulted in toxin production. In some of these experiments, however, samples taken at different times in the growth of the culture showed some evidence of a slight rise and then a destruction of toxin.

DISCUSSION

The work described shows that the specific toxin of *B. anthracis* present in the plasma of guinea-pigs dying of anthrax has at least two components (Factors I and II). They have a marked synergic action on each other in both the skin test and in the mouse lethality test. There does not seem to be a large excess of one or other of the two components in the plasma since addition of the separate components to the toxic plasma does not increase its activity.

Factor I of the toxin was quickly deposited by ultracentrifugation under conditions described for the separation of the immunising aggressin (Fraction Y) in a previous paper (Smith and Gallop, 1956). When purified by repeated ultracentrifugation, its ultracentrifuge diagram was almost identical with that of the latter preparation. The two preparations also had similar immunising and antiphagocytic properties but they differed in two important respects. First, Factor I was toxic when recombined with Factor II but Fraction Y was not. Second, Factor I had virulence-enhancing activity, as had the original toxin (and plasma/exudate) but Fraction Y had not. Fraction Y is however, closely connected with the toxin since a hyperimmune serum prepared against it neutralised the toxin. It seems highly probable therefore, that Fraction Y is a "toxoided" form of Factor I. In fact, when purified Factor I was treated with barium acetate and ethanol (as was Fraction Y), its power of recombining with Factor II to form a toxic mixture was largely destroyed (*cf.* the action of barium acetate and ethanol on the original toxin (Smith *et al.*, 1955)). The fact that at least one component of the anthrax toxin is an active aggressin means that the aggressive action of *B. anthracis* in the early stages of anthrax is to some extent due to the toxin which later in the disease acts to kill the host.

Factor II of the toxin remains in the supernatant after ultracentrifugation. Its synergic action with Factor I cannot be simulated by polyglutamic acid or by its own ultrafiltrate. An antigen produced *in vitro* can replace Factor II in the synergic mixtures tested for mouse lethality and oedema-producing activity provided sufficient of Factor I is present. However, two findings show that *in vitro* antigen is not identical with Factor II. First, addition of the *in vitro* antigen to Factor II forms a synergic not an additive mixture in a mouse lethality test although the skin reaction is unchanged. Second, whereas Factor II acts synergically with I even when the latter is in a non-reacting concentration, the *in vitro* antigen is only capable of increasing the effect of a dose of Factor I which already produces a slight skin reaction independently.

Further purification of the two components of the anthrax toxin is proceeding and the relationship, if any, of Factor I with the S20 component of normal serum is being investigated.

It would have been convenient for chemical fractionation to have been able to produce the anthrax toxin *in vitro* but all attempts to do this failed. The failure of experiments involving the use of ordinary laboratory media was not surprising in view of the unsuccessful attempts in the past (Eurich and Hewlett, 1930 ; Sobernheim, 1931 ; King and Stein, 1950).

It is interesting that the blood of an infected guinea-pig at the early bacteraemic stage (Keppie *et al.*, 1955) which 8–10 hr. later *in vivo* would be toxic, produces no toxin when removed and incubated *in vitro* although the rate of growth and extent of capsulation of the organisms were almost equal to those obtained *in vivo*. Hence the particular factor of the host environment responsible for toxin production remains undetermined.

SUMMARY

The specific toxin of *B. anthracis* in the plasma of guinea-pigs dying of anthrax has at least two components which form a synergic mixture.

One component (Factor I) deposited quickly by ultracentrifugation has been partially purified ; it is an immunising antigen and an aggressin. The immunising aggressin (Fraction Y) separated from plasma/exudate (see Smith and Gallop, 1956) is probably this component which has been " toxoided " by the extraction process. A second component (Factor II) remains in the supernatant after ultracentrifugation. The immunising antigen purified from *in vitro* sources by Strange and Belton (1954) is connected with Factor II of the toxin but is not identical with it.

Polyglutamic acid produced by *B. anthracis in vivo* is not a component of the toxin.

Attempts to prepare the anthrax toxin *in vitro* using conditions approaching those occurring *in vivo* have failed.

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THE NEUROTOXIN OF *SHIGELLA SHIGAE*. A COMPARATIVE STUDY OF THE EFFECTS PRODUCED IN VARIOUS LABORATORY ANIMALS

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THE paralytic effect on rabbits of the exotoxin of *Shigella shigae* has led many observers to class it as a neurotoxin. It does not appear, however, that its effects are due to a primary attack on nerve cells (Howard, 1955), but rather to be secondary to damage to the endothelium of small blood vessels in the neuraxis (Bridgwater, Morgan, Rowson and Payling Wright, 1955). This latter conception is supported by the present comparative study of the effects of the toxin on various laboratory animals, for, in marked contrast to the neurotoxins of *Clostridium tetani* and *Clostridium botulinum*, greatly varying syndromes of intoxication can be produced in different species which are not always of neurological type.

MATERIALS AND METHODS

Toxin

Two preparations of toxin were tested. Firstly, the same sample of the purified neurotoxin of van Heyningen and Gladstone (1953) was used as had been employed by Bridgwater *et al.* (1955). Secondly, a less highly fractionated preparation containing 100 mouse LD_{50} per ml. was also used in some earlier experiments. No qualitative difference in biological activity was detected between these two materials in any animal species. A preparation of the endotoxin (somatic antigen) of *Sh. shigae* with a mouse LD_{50} of 100 μg. was kindly given by Dr. D. A. L. Davies of the Microbiological Research Department, Porton.

Antitoxin.—The same preparation was used as previously described by Howard (1955).

Animals.—The mice used were of the Webster strain and weighed 20–22 g. The rats were of an albino strain weighing 170–180 g. Hamsters were used weighing approximately 120 g. Guinea-pigs weighed 750–830 g. and albino rabbits 2–3 kg.

Operative techniques

Mice and rats were injected intravenously into a tail vein, using a volume not exceeding 0·2 ml. and 1·0 ml. respectively. Hamsters were injected intraperitoneally. Both guinea-pigs and rabbits were injected intravenously, the former into a saphenous vein and the latter into an ear vein.

Histological methods

The material from each species was fixed in formol saline as soon after slaughter of the animal as possible. Since removal of the unfixed central nervous systems of these small animals is extremely difficult without damaging them, the skull was opened and the muscles stripped off the spinal column to allow preparatory penetration of the fixative. The brain and spinal cord were removed when adequately hardened. Brains were cut into coronal slices about 3–4 mm. thick and all the slices embedded. The whole spinal cord was likewise embedded after

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cutting into convenient lengths and sectioned longitudinally. Sections from the paraffin-embedded material were stained with haematoxylin and eosin and Mallory's trichrome stain; for C.N.S. tissue Glees and Marsland's stain for axons and Loyez' stain for myelin sheaths were also employed.

RESULTS

The Intoxication Produced in Rats

Rats are fairly resistant to the exotoxin, being about seven times more so than mice per unit weight. The first signs of intoxication appeared at least 18 hr. after intravenous injection when the animals appeared immobile with ruffled fur. Diarrhoea developed shortly afterwards, usually accompanied by oral regurgitation of stomach contents. The flaccid paralysis or convulsive episodes, characteristic of the effect of this toxin in rabbits and mice respectively, were never observed. Rats usually died not less than 24 hr. after the earliest appearance of gastro-intestinal signs, without showing any other characteristic development.

At necropsy the most striking feature was gross dilatation of the stomach, which was filled with liquid. The small intestine was similarly dilated and showed areas of vascular damage in the middle and lower thirds; this was presumably responsible for the condition of ileus. In some rats this consisted of areas of intense congestion, while others showed diffuse ecchymotic haemorrhages in the submucosa or more extensive haemorrhagic necrosis. The large bowel was empty and its wall was normal. The survival periods and degree of bowel damage in some of the rats examined are shown in Table I. Severely intoxicated rats killed several hours before death was expected showed the same severe dilatation of the stomach, but the wall of the ileum, although very oedematous, did not appear haemorrhagic. The rat given the lowest killing dose (30 mouse LD_{50}) survived for a long period (130 hr.), and at necropsy the cause of death was not apparent.

TABLE I.—*The Effect of Shiga Neurotoxin in Rats*

Intravenous dose of toxin (Mouse I.P. LD_{50}).	Survival period (hr.).	Stomach.	Severity of lesions in small intestine.
100	36	Grossly dilated	++
100	45	" "	++++
60	47	" "	+++
60	50	" "	+
50	55	" "	+++
50	60	" "	++
30	130	Normal	—
15	Survived	"	—

In conformity with the absence of nervous signs in this species, no lesions could be detected histologically in the C.N.S. of animals surviving 50–60 hr. Microscopical examination of lesions in the small intestine showed superficial desquamation of the mucosa with outpouring of polymorphonuclear leucocytes and extravasation of red blood cells (Fig. 1). At an earlier stage, oedema of the mucosa and submucosa with the escape of a few red cells was found (Fig. 2), without the destructive changes in the mucosa noted above. Heart, lungs, liver and stomach showed nothing abnormal.

These lesions are clearly related to the action of the exotoxin, for complete protection is afforded against 100 mouse LD_{50} by giving 100 units antitoxin I.V.

15 min. previously. Furthermore, heating at 100° for 2 min. renders the toxin quite inactive. A preliminary experiment has confirmed that as with the mouse (Howard, 1955) and rabbit (Payling Wright, personal communication), rats could not be protected against the effect of the toxin by even a large intravenous dose of antitoxin if the latter was delayed for as little as 30 min.

Finally, a differing syndrome is produced by the endotoxin of *Sh. shigae*. Two animals, each given 2·5 mg. purified endotoxin intravenously, became ill after a much shorter period than with the neurotoxin and died after 18 and 30 hr. respectively. At post-mortem the stomach appeared normal and bowel changes were restricted to congestion of the small intestine, part of a more generalised congestion involving especially the liver and spleen.

The Intoxication Produced in Hamsters

Hamsters are quite sensitive to Shiga exotoxin injected intraperitoneally, being killed by as little as 0·3 mouse LD_{50}. As with other species, a long delay (at least 30–40 hr.) preceded the onset of symptoms. The intoxication syndrome was less characteristic than in rabbits, mice or rats. Following this lag period the hamsters remained quiet with ruffled fur and not eating. Within a few hours, the condition was that of general weakness and the animals walked unsteadily with a coarse tremor. No signs of paralysis, convulsion or diarrhoea were ever observed. Hamsters usually succumbed about 6 hr. after these signs had developed fully (see Table II).

TABLE II.—*The Effect of Shiga Neurotoxin in Hamsters*

Intraperitoneal dose of toxin (Mouse I.P.LD_{50}).	Survival period (hr.).	Pleural effusion + oedema of lungs.	Other features.
100	60	−	—
50	45	−	—
20	42	+	Congestion of liver.
15	49	−	—
10	73	+	Congestion of liver.
10	75	+	„ „ „
2·5	68	−	Congestion and ecchymoses in caecum.
0·5	64	+	Congestion of liver.
0·3	204	−	Congestion of small intestine and liver.
0·1	Survived	−	—

The post-mortem findings were not uniform. The most prominent feature, in about half the animals, was a large bilateral serous pleural effusion associated with congestion and oedema of the lungs. The liver was frequently congested, the spleen and kidneys less so. The bowel was usually normal except in one case where a few ecchymoses were present in the caecum and in another where the ileum showed generalised congestion. In three animals no gross abnormality was detected.

Except for a small and probably incidental granuloma of uncertain nature in the spinal cord of one animal, histologically the central nervous tissues were clear of any pathological change. The fairly regular occurrence of ataxia and coarse tremor as the main feature of the terminal illness in this species demanded a close examination of the cerebellar and vestibular pathways. No early degenera-

tive changes, however, in nerve cells or axis cylinders were found, though the possibility that functional disturbance might occur in the absence of visible changes cannot be excluded. In the lungs the only discernible microscopic changes were accumulation of fluid in the alveolar spaces, particularly in a zone beneath the pleural surface (Fig. 3), and oedema fluid in the adventitia of the pulmonary veins (Fig. 4). Accompanying this in each case were small numbers of extravasated red blood cells and a few reacting mononuclear cells.

The Effect of Shiga Neurotoxin on Guinea-pigs

It has been widely found in the past that guinea-pigs are not susceptible to the exotoxin of *Sh. shigae*. We have re-examined this finding, firstly by injecting large doses of toxin intravenously, a route by which any potential lethality should be enhanced. None the less, three animals survived 200 mouse LD_{50} without any disturbance. Secondly, these animals were killed after five days and examined histologically, since minute lesions could conceivably be present in the neuraxis without producing signs. No histological abnormality was detected in the brain, spinal cord or viscera.

In contrast, the guinea-pig is normally susceptible to the endotoxin of *Sh. shigae*. Two animals were killed by 3 mg. in 24 hr. and at necropsy showed congestion of the bowel, spleen, liver and mesentery, as observed by other workers.

The Lesions Produced by Shiga Neurotoxin in Mice

Earlier reports have frequently disagreed as to the effect of this toxin in mice, but the syndrome of flaccid paralysis and tetanic convulsions (Howard, 1955) suggests nervous involvement. No macroscopic lesions have been observed at post-mortem, but distinct changes were visible histologically in the brain and spinal cord of all mice examined which had received 0·75–5·0 LD_{50} toxin intravenously (Table III). The change was focal and scattered through the neuraxis more or less at random (Fig. 5–7), but with some predilection for the brain stem and the spinal cord. The cerebral cortex did not escape involvement, for occasional small foci were encountered here, but none were seen in the cerebellum. In sections from severely ill animals, sacrificed 48 hr. after receiving 5 LD_{50} toxin, the chief feature was multiple fine vacuolation and general pallor of the affected areas. The vacuoles contained unstainable material and appeared to be displacing the normal structures. In these regions an occasional nerve cell showed early anoxic

TABLE III.—*Distribution of Lesions in the Central Nervous System of Mice following the Intravenous Injection of Shiga Neurotoxin*

Intravenous dose of toxin (LD_{50}).	Survival period (hr.).	Nervous lesions demonstrated.		
		Midbrain.	Medulla.	Spinal cord.
5	48	++	+	++
5	48	−	−	++
5	48	++	+	++
5	48	−	++	+
1·0	90	+	−	+
1·0	96	+	+	+
0·75	96	+	−	+
0·75	108	−	−	+

damage, notably nucleolar swelling, but more marked expressions of damage were not encountered. Except for the very occasional presence of an activated microglial nucleus, no further reaction to the vacuolation was found. Haemorrhages were not seen.

In order to prolong survival, some further mice were given only 0·75–1·0 LD_{50} toxin and were examined 90–108 hr. after inoculation. The foci were in general noticeably smaller in size than those produced by the larger dose examined at 48 hr. At 90 hr. an occasional ischaemic neurone was seen in the oedematous zones, but no glial response. In the mouse that survived 108 hr., the longest in this series, ischaemic neurones were numerous in the spinal cord lesions and microglial activation was apparent. Again, no haemorrhages were encountered in the nervous tissue foci, and at no time was any structural change seen in the blood vessels.

The Production of Extra-neural Lesions in Rabbits

The profound neurological effect obtained in rabbits has tended to obscure the fact that, although less constant, intestinal changes are frequently present in intoxicated rabbits. Several early workers (*e.g.*, Flexner and Sweet, 1906) found that the wall of the caecum showed changes varying from gelatinous oedema to haemorrhagic necrosis; although at this period very crude preparations were used, similar findings were recorded by Istrati (1938) and Boivin (1940) using trichloracetic acid-precipitated exotoxin. Such caecal lesions appeared in less than half the latter's rabbits, but Steabben (1943) found that they occurred more consistently if the toxin was given intracutaneously rather than intravenously. To exclude the possibility that these changes were a manifestation of Shiga endotoxin in the preparations, we have examined the purified toxin of van Heyningen and Gladstone for its ability to produce such caecal lesions.

With small doses (1–2 rabbit LD_{50} I.V.), the animals' faeces were normally formed and the caecum appeared normal at post-mortem examination. Forty-eight hours after a dose of 20 rabbit LD_{50} I.V., the animals constantly showed diarrhoea of varying severity. At necropsy severe gelatinous oedema of the caecum was found, the mucosa frequently being speckled with petechial haemorrhages. In a few instances, widespread confluent haemorrhage and superficial

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EXPLANATION OF PLATES

FIG. 1.—Rat ileum showing superficial necrosis of villi and polymorphonuclear leucocytic infiltration of mucosa. Haematoxylin and eosin. × 107.

FIG. 2.—Rat ileum with pronounced oedema of the submucosa and intact mucosal layer. A few red cells and mononuclear cells lie free in the exudate. Haematoxylin and eosin. × 80.

FIG. 3.—Hamster lung with alveoli filled with protein-containing fluid. Haematoxylin and eosin. × 165.

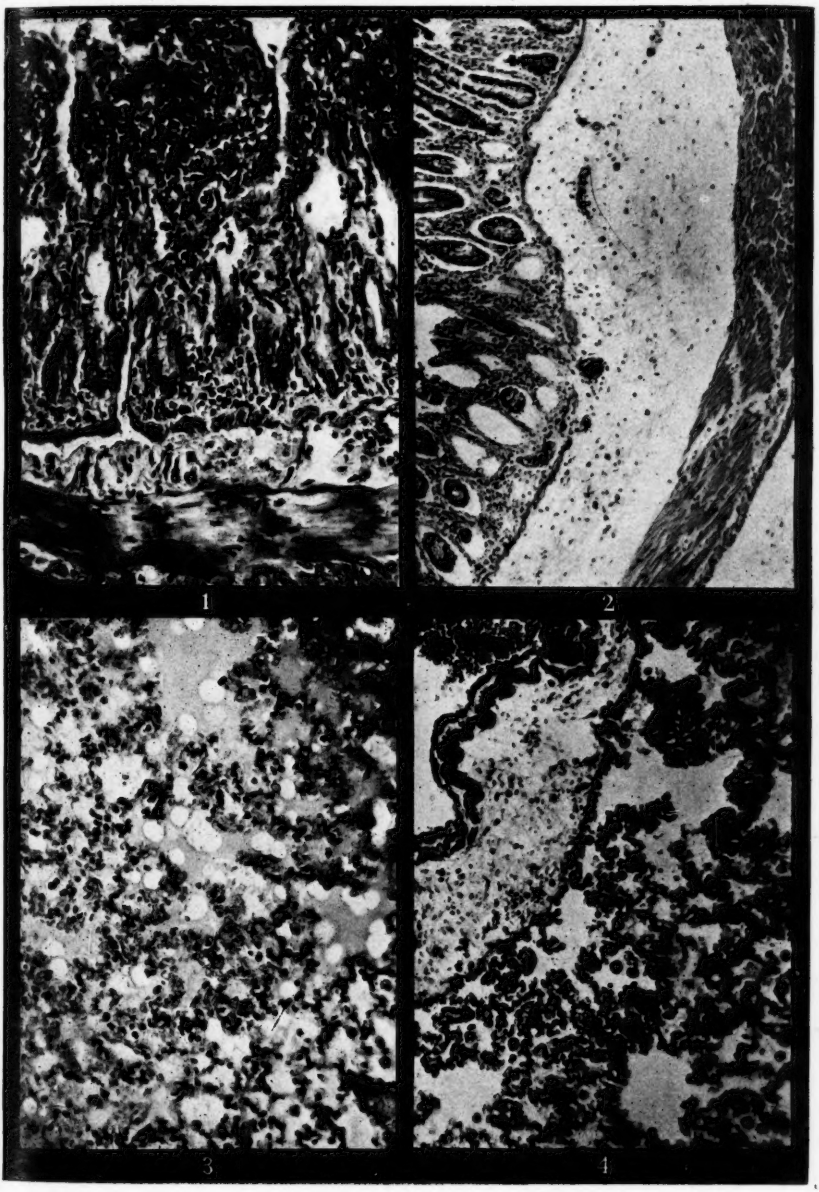
FIG. 4.—Hamster lung. Oedema fluid containing a few red cells and mononuclear cells in the space around a pulmonary vein. Haematoxylin and eosin. × 165.

FIG. 5.—Mouse upper brain stem. A large area of oedema containing degenerating neurones. Mallory's trichrome stain. × 110.

FIG. 6.—Mouse medulla. Sharply circumscribed focus of damage near the meningeal surface. Mallory's trichrome stain. × 110.

FIG. 7.—Mouse spinal cord. Two unequal areas of vacuolation in the grey columns. Mallory's trichrome stain. × 110.

FIG. 8.—Rabbit caecum. Severe oedema of submucosal connective tissue and of the mucosa with diffuse haemorrhage in part of the latter. Haematoxylin and eosin. × 19.



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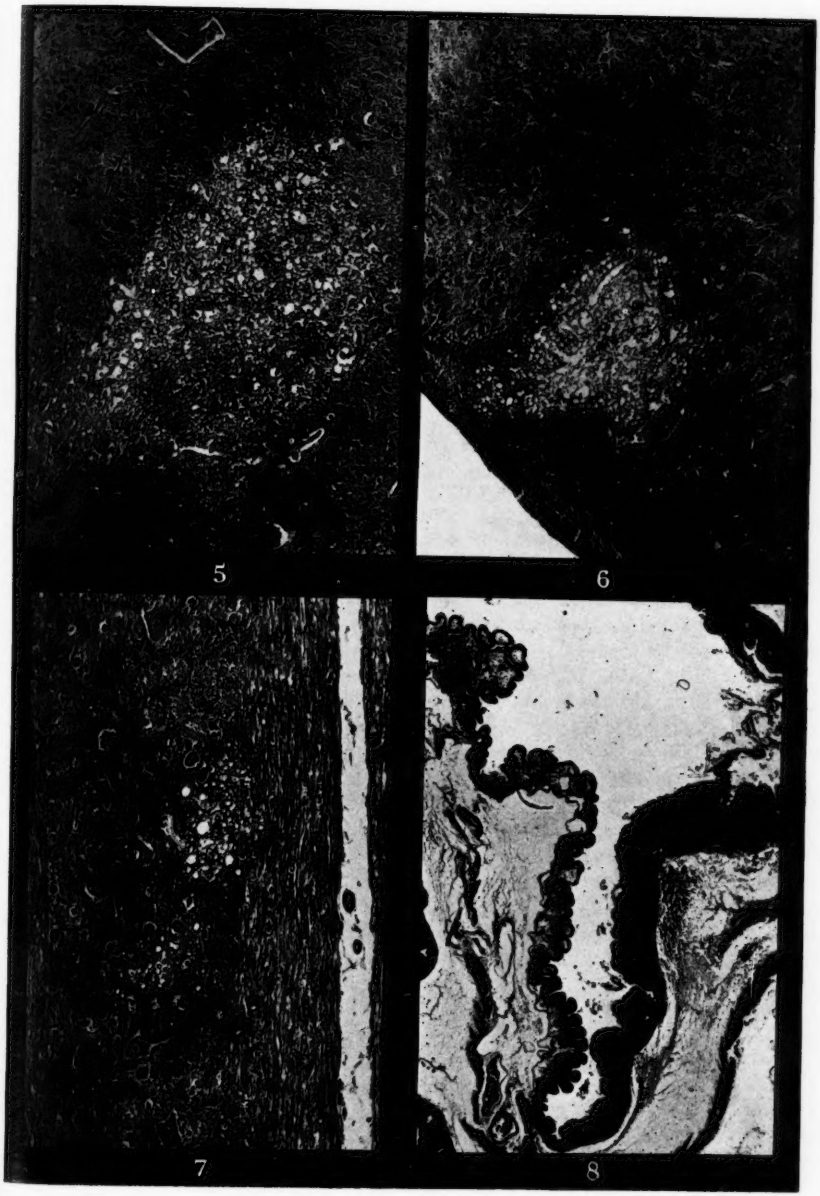

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mucosal necrosis occurred. Histologically, the most striking change was severe oedema of the loose submucosal areolar tissue and of the mucosa (Fig. 8). Extravasation of red blood cells and a few infiltrating inflammatory cells were also present. No alterations in the blood vessels to account for these changes were seen.

DISCUSSION

Since the demonstration by Dopter (1905) that dysenteric toxin caused serious lesions in the spinal cord of rabbits, the view has become general that the exotoxin of *Sh. shigae* is primarily a neurotoxin ; and little attention has been paid either to the changes occurring in other organs or the variation in response in the different species. The histological studies of Kishida (1953) and Bridgwater *et al.* (1955) on the rabbit have cast considerable doubt on the specifically neural affinity of the " neurotoxin " and have indicated that damage to nervous tissue is mediated through primary damage to its blood vessels. Similarly, in the mouse the development of paralysis of the hind limbs and convulsions is probably referable to focal areas of oedema and thus to local disturbances of nutrition in the nervous parenchyma rather than to primary neural damage. Histological evidence of injury to the nerve cell in this animal does not appear until many hours after the foci of oedema have been evident. This dependence of the nervous lesions on earlier vascular damage is analogous to that outlined for the rabbit by Bridgwater and his colleagues. The more widespread occurrence of lesions throughout the neuraxis is, however, comparable to the findings of Kishida (1953) in the monkey.

In contrast, no nervous lesion can be demonstrated by careful histological examination in the guinea-pig, the hamster or the rat, although the hamster shows some curious terminal ataxic signs which may not be essentially neuraxial in origin. The hamster, however, frequently shows clear pleural effusions at death. Similar effusions were occasionally observed by Istrati (1938) in the rabbit, but not apparently by Bridgwater *et al.*, or by us. Coincidentally, areas of perivascular oedema with escape of red blood corpuscles were often present in the lungs of these affected hamsters. The latter change was reminiscent of the severe perivascular oedema which is a feature of guinea-pigs' lungs after the intravenous injection of Forssman antigen.

The changes in the alimentary tract support the belief that the exotoxin of *Sh. shigae* acts essentially on blood vessels, although there is considerable species variation in the susceptibility of the intestine to the toxin. Boivin (1940), in a comparative study of the effects of the exo- and endo-toxins of *Sh. shigae*, noted that although the endotoxin caused intense diarrhoea in mice, rabbits and guinea-pigs, the exotoxin produced bowel lesions in 8 out of 18 rabbits but none in mice or guinea-pigs. In rabbits the small blood vessels of the intestinal tract are considerably less sensitive that those in the spinal cord enlargements. On the other hand, the rat shows no nervous lesions even with 100 mouse LD_{50} toxin, but oedema and petechiae are constantly present in the bowel both with this dose and with 50 mouse LD_{50}. The changes are similar to those in the rabbit, namely oedema, haemorrhages into the mucosa and later focal necrosis of the mucous membrane.

When the lethal doses of toxin per unit weight of these various species are compared, great differences in susceptibility are apparent (Table IV). Furthermore, no correlation emerges between this species resistance series and the distribution of tissue damage.

TABLE IV.—*The Relative Resistance of Various Animal Species to Shiga Neurotoxin*

Resistance expressed as the ratio obtained by comparing the species LD_{50} (μg. toxin per kg. body weight) with that for the rabbit.

Rabbit	1
Monkey (*Macacus mulatta*)	5
Hamster	40
Mouse	700
Rat	5,000
Guinea-pig	>10,000

(Calculated from personal observations and incorporating data from van Heyningen and Gladstone (1953) and Branham, Habel and Lillie (1949).)

Involvement of the nervous system is very rare in human Shiga dysentery; only 2 instances in 3800 cases were described by Dumas in 1938. Such occurrences have been described as "peripheral neuritis", a recognised, but ill-defined, complication of many other infectious diseases. It is probable, therefore, that while the nervous system of man is as resistant to the neurotoxin as that of the rat, the possibility that some extra-neural site in the body may be affected cannot be excluded.

SUMMARY

A toxicological and morphological study of the effects of the exotoxin of *Sh. shigae* upon rats, hamsters, guinea-pigs, mice and rabbits has been made.

Nervous lesions occurred in mice and rabbits, but in none of the other species tested. Oedema and haemorrhages in the gastro-intestinal tract occurred regularly in rats, and in rabbits with high dosage. Hamsters, in contrast, frequently showed pulmonary oedema and pleural effusions as the only changes. Guinea-pigs were completely resistant to the toxin.

For all these lesions a basically vascular origin is suggested, and no relationship between susceptibility to the lethal effect of the toxin and localisation of the damage was apparent.

We wish to express our thanks to Dr. W. E. van Heyningen, Dr. D. A. L. Davies and Miss Mollie Barr for preparations of toxin and antitoxin, and to Professor G. Payling Wright for his interest in this paper.

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MYOCARDIAL NECROSIS IN THE POTASSIUM-DEPLETED RAT: A REASSESSMENT

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In 1937, Schrader, Prickett and Salmon reported that myocardial necrosis occurred in rats placed on a potassium-deficient diet. They found patchy, complete destruction of muscle, more in the left than the right ventricle. The valvular tissue was seldom involved, the auricles never. The necrotic muscle was replaced by scar tissue and numerous phagocytic cells.

In reports published since then, there has been considerable difference of opinion as to the histological features and localisation of the lesions. Thomas, Mylon and Winternitz (1940) noted that lesions were most marked subendocardially, that there was no involvement of vessels, that the cellular exudate was predominantly macrophage in type and that deficiency of B-complex vitamins aggravated the lesions. This last point was refuted by Darrow and Miller (1942) who found that thiamine and pyridoxine supplements had no protective effect in potassium depletion. Follis, Orent-Keiles and McCollum (1942) reported that while lesions were frequently subendocardial, they never involved the endocardium itself. Kornberg and Endicott (1945) stated that auricular lesions did occur. Lesions produced by high dosage of desoxycorticosterone acetate, and described as "rheumatic fever" by Selye and Pentz (1943) were shown by Peschel, Black-Schaffer and Schlayer (1951) to be due to potassium deficiency. These latter authors noted the presence of numerous Anitschkow cells, and confirmed the occurrence of auricular lesions. French (1952) was the first to point out that the necrosis of potassium deficiency differed from that of ischaemia or cauterisation in that the lesions remained vascularised throughout, and showed no destruction of the connective tissue framework.

Similar lesions have been described in the hearts of mice (Liebow, McFarland and Tennant, 1941), pigs (Thomas *et al.*, 1940), cats but not dogs (Darrow and Miller, 1942) and humans (Goodof and MacBryde, 1944; Rodriguez, Wolfe and Bergstrom, 1950; Perkins, Petersen and Riley, 1950; Luft, Ringertz and Sjögren, 1951). McAllen (1955) was the first to suggest that massive myocardial scarring in the human might result from potassium deficiency.

During a personal study of other aspects of potassium depletion in rats, a cardiac lesion was regularly found which did not seem to correspond with the available descriptions. Because of this, an attempt was made to correlate the histological findings in the heart of the potassium-deficient rat with changes in the electrocardiogram and tissue chemistry. Only the histological findings are presented here. In all, 69 potassium-deficient rat hearts were studied, after varying periods on the potassium depletion regimen. Some animals were repleted with potassium before being sacrificed, to observe the effect on the lesions.

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MATERIALS AND METHODS

Adult male albino rats averaging 180 g. in weight were placed on a prepared casein-dextrin-arachis oil diet with added vitamins, as described by Cooke, Segar, Cheek, Coville and Darrow (1952). Duplicate analysis showed that this diet contained 5 mg. potassium per 100 g. dry feed. Average consumption with liberal feeding was just under 40 g. per kg. rat per day, so that each animal received considerably less than 1 mg. of potassium per day. The only drinking fluid was a mixture of 0·25 per cent $NaHCO_3$ and 0·25 per cent NaCl in water. Each animal was given 2 mg. DOCA intraperitoneally daily for the first week. Sodium sulphonic resin in the sodium cycle ("Resonium A", Bayer), 2·5 g. per 100 g. dry feed, was mixed with the diet each day, giving approximately 1 g. resin per kg. rat per day; this is comparable to human dosage levels.

Marked and rapid potassium depletion was produced by this regimen, spontaneous deaths occurring from the 20th day onwards. Animals were sacrificed by ether anaesthesia and exsanguination from the inferior vena cava. This provided blood for analysis and relatively blood-free organs for histological study. As a routine, a 2 mm. cross-section was cut from the ventricles mid-way between apex and base. This was fixed in Helly's fluid, the rest of the heart being placed in 15 per cent formol-saline-calcium for further study. After embedding in paraffin wax, sections were cut at 5μ and stained with haematoxylin-eosin, Mallory's azo-carmine, Mallory's phosphotungstic acid-haematoxylin, Best's carmine, periodic acid Schiff, Sudan III and IV and Gordon and Sweet's modification of Wilder's reticulin method.

RESULTS

While there are great variations in the appearance of the lesions at various stages of potassium depletion, all can be related to three basic processes. These are:

1. Degeneration and death of muscle fibres.
2. Phagocytosis of necrotic material.
3. Collapse of the supporting stroma.

Important negative features are the absence of capillary ingrowth, though not of capillary reaction, absence of a fibroblastic response and virtual absence of polymorphonuclear leucocytes.

EXPLANATION OF PLATES

FIG. 1.—Hypokalaemic necrosis. An early lesion, showing varying degrees of loss of striations, but no cellular infiltration. Seventh day of depletion. Mallory's phosphotungstic acid haematoxylin. × 250.

FIG. 2.—A small lesion showing cellular infiltration. Arrows point to some of the prominent capillary nuclei. (See also Fig. 4.) Twelfth day of depletion. Haematoxylin and eosin. × 265.

FIG. 3.—A large subendocardial lesion in an example of "hypokalaemic necrosis." (Also see Fig. 5.) Twentieth day of depletion. Haematoxylin and eosin. × 220.

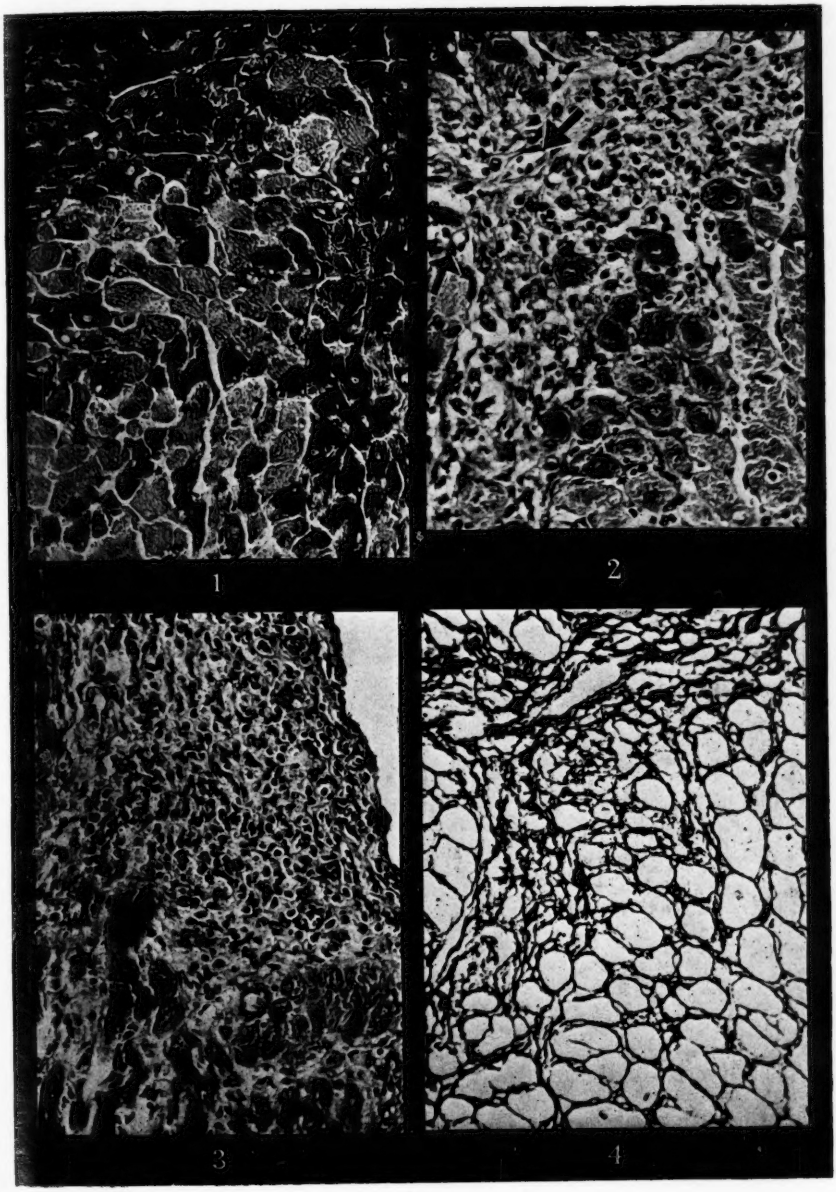
FIG. 4.—Reticulin pattern of the lesion shown in Fig. 2. Note there is compression and collapse, but little distortion of the pattern. Wilder's reticulin method. × 250.

FIG. 5.—Reticulin pattern of the lesion, Fig. 3. There is irregular collapse and compression, but little destruction of the pattern. Wilder's reticulin method. × 400.

FIG. 6.—A severely affected heart showing marked separation of the muscle bundles. Arrows point to grey necrotic foci where phagocytosis is almost complete. Dark areas are foci of more recent necrosis. The black in the upper left-hand corner is artefact. There is very little normal myocardium. Twenty-fifth day of depletion. Azo-carmine. × 16.

FIG. 7.—Reticulin pattern in a lesion of at least 6 weeks' duration. The circular structures in the lesion are capillaries. The faintly-staining lines show the staining reactions of collagen, the dark lines those of reticulin. Sixty-three days after the start of depletion, 42 days after repletion. Wilder's reticulin method. × 300.

FIG. 8.—Accumulation of glycogen in muscle fibres bordering a necrotic focus. Arrows point to some of the larger deposits. Twenty-second day of depletion. Best's carmine. × 480.



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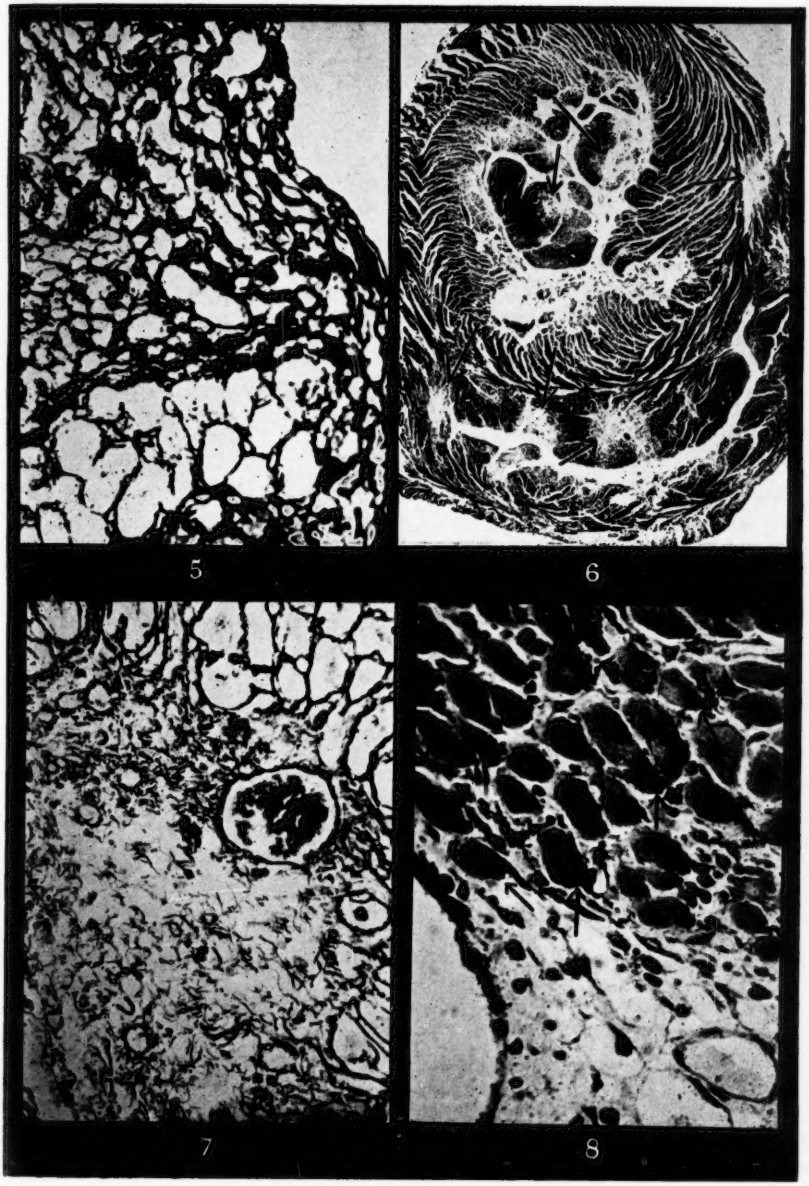

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The various stages of the lesion, and the sequence in which they occur, will be described, and then the analogy to other commoner lesions will be traced. It should be emphasised that fresh lesions continue to appear throughout potassium depletion so that all stages may be seen in a heart examined after, say, 20 days of depletion. Also, since serial biopsy is impossible, any estimate of the duration of the various stages can be only approximate.

The earliest stage of the lesion, at the end of the first week of depletion, can be detected only by special stains. It consists of some swelling of the muscle fibres and gradual loss of striations (Fig. 1). Following this the nucleus usually undergoes karyolysis, fading and disappearing. Pyknosis is also seen but its frequency is more difficult to estimate, since the pyknotic nuclei of inflammatory cells can be seen in degenerate muscle fibres from an early stage. Some of the neighbouring fibres show clear vacuoles, as described in McAllen's Case 1 (1955). These vacuoles do not contain fat or glycogen.

Soon after (by the 8th day of depletion), or even simultaneously with this loss of striations, cells accumulate round the affected fibres; this is the first stage which can be detected by routine haematoxylin-eosin staining (Fig. 2). The cells consist mainly of tissue macrophages, but sarcolemma nuclei can also be seen, and an occasional polymorph. The cells have been described as being predominantly polymorph in type, but on close examination this is obviously not so. Even in the largest and acutest lesions ("hypokalaemic myocarditis", Rodriguez *et al.*, 1950) the same type of inflammatory cell is seen (Fig. 3). What appears in these cases to be a widespread polymorph infiltration of the rest of the myocardium, can be seen on closer examination to consist of darkly-staining tissue macrophage nuclei, very prominent capillary endothelial nuclei and only an occasional polymorph or eosinophil. These tissue macrophages have also been described as "Anitschkow myocytes" (Peschel *et al.*, 1951), but while their derivation may be the same, the typical Anitschkow nuclear pattern is seen in only a small proportion.

In every report the presence of fibroblasts has been noted. Many of the nuclei appear to resemble fibroblastic nuclei, although the more closely they are examined the more of them are found not to have the typical appearance of fibroblasts. Moreover, as the lesion progresses, there is no evidence at all that they lay down collagen, nor are they transformed into fibrocytes. It is felt that the term "fibroblast" should be reserved for cells which have the function as well as the appearance of fibroblasts.

A most important feature, first noted by French (1952), is that there is no destruction of the connective tissue framework, and this is best demonstrated by reticulin impregnation. There is usually some collapse of the reticulin pattern, even in the early stages, but there is no destruction. This is shown by Fig. 4 and 5, which are the reticulin preparations of the areas shown in Fig. 2 and 3. Collapse of the stroma usually begins while phagocytosis is still active.

When the phagocytic cells disappear (11th day onwards), collapse becomes complete in the smaller lesions and they can no longer be detected by routine staining but azo-carmine shows such a lesion as a bright blue line with thinner lines radiating from each end, the whole sandwiched between bright red surviving muscle fibres. In larger lesions, collapse is delayed, or is only partial, resulting in a network of open spaces with almost no cells. However, a few normal capillaries always traverse such areas. This can be seen from about the 15th day of depletion.

Particularly in relation to large lesions, oedematous separation of the muscle bundles may be very striking (Fig. 6). The degree of separation varies with the acuteness of the lesion and also to some extent with its situation, as those near the endocardium or epicardium more often show it than those in the depth of the muscle. In the most severe cases, of which Fig. 6 is an example, the separation may be generalised.

Another feature which is also more prominent in the larger lesions is the presence of small irregular muscle fibres with prominent closely-set striations, near the edge, and sometimes in the centre, of the collapsed stroma. The appearance suggests that these are possibly regenerating fibres. Apart from this the lesion remains indolent and largely acellular. There is no evidence of capillary budding or ingrowth of new vessels, nor are fibroblast-like cells seen at this stage, which has been found to persist for up to 10 weeks. Gradual disintegration of the reticulin occurs, the strands taking on the staining reactions of collagen. Sometimes reticulin and collagen may be seen in the same fibre, in a way strongly suggesting that reticulin is being converted to collagen rather than that collagen is being laid down from outside the area.

Fig. 7 shows the reticulin preparation of such a lesion, which is at least 6 weeks old, the animal having been given potassium after 3 weeks of depletion to prevent the occurrence of further fresh lesions. The appearance is quite unlike that seen 6 weeks after ischaemic necrosis.

It seems that the variation in the time required for the lesion to disappear—4 days to at least 10 weeks—depends principally on the size of the lesion. However, no matter how long it persists, a dense scar is never formed. Indeed, examination of hearts up to 4 months after correction of the potassium depletion suggests that all these lesions will ultimately disappear, given sufficient time, but it would be difficult to prove this conclusively.

Since the hypokalaemic lesion differs in aetiology, pathogenesis, healing and end-result from the post-necrotic or post-inflammatory scar, it is felt that confusion might be avoided to some extent by not using the term "scar" at all for the former lesion.

A striking feature of the hypokalaemic lesion is the accumulation of glycogen in damaged muscle fibres. This is not peculiar to hypokalaemia as it can be seen in relation to foci of ischaemic necrosis, but it is unusually widespread in severe potassium depletion. It rapidly clears on giving potassium, except in the cells bordering necrotic foci, where it may persist for weeks (Fig. 8). This glycogen stains very poorly with Schiff's reagent, but it is glycogen, since it is removed by prior treatment with diastase. In the macrophages found in the lesions, on the other hand, material is found which stains red with Schiff's reagent and with Best's carmine, and this is not all glycogen, since only some of it is removed by diastase. The suggestion that this represents the removal of excess ground-substance (French, 1952) seems logical. The amount of glycogen found in the muscle-fibres varies greatly, from a few granules to a mass filling half the cell, but it is always in excess of the amount to be seen in normal cardiac muscle. In view of the known metabolic effects of potassium, this probably represents an accumulation of glycogen which the cell is unable to metabolise, but why it should persist in apparently normal cells which happen to be near a necrotic focus is not evident.

The position as regards accumulation of fat is very different. Deposits of fat were infrequent, never gross, and bore no relationship to the necrotic foci.

Although a specific search was made, no lesion could be found in skeletal muscle, which could be attributed, unequivocally, to potassium deficiency.

DISCUSSION

The present study confirms that lesions occur in the auricles, though they are small and unimpressive. Lesions are seen throughout the ventricles, but the largest are subendocardial, especially in the papillary muscles and trabeculae. This would seem to indicate either that local strain and trauma determine the localisation of the necrosis, or more probably that they aggravate the lesion once it is established. Variation in local blood supply, however, cannot be excluded as a factor. Large lesions are less common subepicardially and distinctly uncommon in the depth of the muscle.

The statements of Follis *et al.* (1942) that the lesions never involve the endocardial surface, and that thrombi are not seen, were confirmed. The way in which intact endocardium covers large areas of completely necrotic muscle is striking. As French (1952) observed, such endothelium is much more cuboidal and crowded together than is usual in the endocardium. If the mechanism involved in this necrosis of muscle is considered, it is not surprising that the endothelium should survive.

It has been shown that the supporting reticulin network remains intact, and the same holds for the endothelial basement membrane. Since connective tissue has a very low potassium content, almost identical with that of extra-cellular fluid, there is no real reason why it should be sensitive to potassium lack. The nutritional requirements of the endothelium of endocardium and capillaries are probably of the same order, and survival of normal capillaries in the middle of necrotic foci suggests that their potassium requirements are not great.

On the basis of the alkaline phosphatase reaction of the capillaries, French (1952) concluded that there was definite evidence of capillary ingrowth into the necrotic foci. However, in the present study no unequivocal capillary budding or ingrowth was seen, but abnormally prominent capillary endothelial nuclei were seen throughout the myocardium in the more acute lesions, and it was considered that the appearance of the capillaries in and around necrotic foci was due to hyperplasia of pre-existing vessels, not to newly-formed vessels.

The conflict between Thomas *et al.* (1940) and Darrow and Miller (1942) as to the value of B-complex vitamins in protecting the myocardium against the effects of potassium deficiency, was not specifically investigated. However, the use of double the amount of B-complex vitamin supplement recommended by Cooke *et al.* (1952) did appear to reduce both the number and extent of the lesions.

The Analogy between Hypokalaemic Necrosis and Other Lesions

As potassium depletion progresses, there is impairment of heart-muscle function. This is shown by changes in the electrocardiogram (low voltage, disappearance of T-waves and spreading of the QRS complex) and by the intracellular accumulation of glycogen. Why this proceeds in places to necrosis is not evident, although local trauma may play a part. For reasons already discussed the connective tissue and blood vessels are not visibly damaged and the reaction to the necrotic muscle is low grade and purely cellular. For this reason, if muscle

regeneration were sufficiently active, " *restitutio ad integrum* " would be theoretically possible. As it is not, the lesion can heal only by collapse and condensation of the connective tissue framework, with eventual reabsorption as a long-term sequel. Potassium repletion accelerates repair and prevents the development of fresh lesions but has no other obvious effect.

An analogous process is seen in mild cases of infective hepatitis. Here also the connective tissue framework is intact, the reaction is cellular rather than vascular and mononuclear rather than polymorph. However, because of the great regenerative powers of the liver cells, restoration of the normal architecture is complete, but this is not possible when the connective tissue framework is also damaged (Dible, McMichael and Sherlock, 1943). Similarly, Oliver, MacDowell and Tracy (1951) stated that in acute tubular necrosis complete restoration of normal architecture and function could occur only if the basement membrane remained intact. By contrast, in toxic necrosis of muscle (*e.g.*, "Zenker's necrosis"), even if the sarcolemma sheath is intact, " *restitutio ad integrum* " occurs only in very small areas ; this is due to the limited regenerative capacity of the muscle cell (Wright, 1950).

It must be emphasised that while potassium deficiency in the rat appears always to produce an identical cardiac lesion, varying only in degree, it is by no means the only process which can produce such a lesion. A very similar picture in the human heart is caused by diphtheria toxin (Warthin, 1924) and it also closely resembles the necrosis caused in the rat heart by mercury and uranium poisoning (unpublished observations). The ischaemic lesion in the human heart, called " myocytolysis " by Schlesinger and Reiner (1955) is also similar.

Reports indicate that potassium depletion produces an essentially similar cardiac lesion in humans, mice, rats, cats and pigs, but not dogs. For this reason, one is reluctant to accept that potassium depletion caused the dense scar found in the second case described by McAllen (1955). Additional atypical features in this case were involvement of the endocardium, mural thrombosis and embolisation, and " only slight concentric diffuse fibrous endarteritis in the larger vessels ". Since the patient had a classical attack of myocardial infarction two years before death, with two subsequent recurrences, ischaemia seems to be a much more probable cause of the scarring than electrolyte disturbance. The presence of relatively normal blood vessels in random sections of the left ventricle does not exclude the possibility of a localised vascular lesion. However, the other scattered areas of " fibrosis " in the left and right ventricles appear to be typical hypokalaemic lesions.

SUMMARY

Cardiac necrosis is found in the potassium-depleted rat from the end of the first week onwards. There is oedematous separation of muscle bundles and accumulation of glycogen in damaged muscle fibres, but connective tissue and blood-vessels are not involved. Inflammatory cells consist almost entirely of macrophages. Healing is by collapse and condensation of the stroma and is complete in the vast majority of cases. Dense scars never result. An analogy is drawn between this lesion and other commoner conditions.

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Mr. J. G. Griffin and his staff prepared the sections, and Miss Klein the photomicrographs.

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THE BASIS OF VIRULENCE IN *PASTEURELLA PESTIS*: THE DEVELOPMENT OF RESISTANCE TO PHAGOCYTOSIS *IN VITRO*

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STUDIES of the comparative behaviour of virulent and avirulent strains of *Pasteurella pestis*, following their intraperitoneal injection into mice, brought to light an important difference between the two types which was not evident from comparative studies *in vitro*. After a short period *in vivo* virulent organisms acquired the ability to resist phagocytosis by mouse polymorphonuclear cells whereas avirulent organisms did not (Burrows and Bacon, 1954*b*). Observations on the behaviour of virulent cells *in vivo* were interpreted to indicate that virulent organisms acquired resistance to phagocytosis through modification of their surface properties and that they exerted an inhibitory action on the polymorphs. Whether or not these two phenomena were determined by a single agency, and whether or not the interpretation of the observations made *in vivo* were substantially correct, demanded a study of the development of resistance to phagocytosis under controlled conditions *in vitro* for satisfactory answer.

In this paper we report attempts to obtain phagocytosis-resistant virulent organisms by treatments *in vitro* and to investigate factors which influence the change from sensitivity to resistance to phagocytosis. Throughout, to avoid monotonous repetition, the phrase "to phagocytosis by mouse polymorphonuclear leucocytes" is to be understood whenever the terms sensitive, sensitivity, resistant and resistance are used.

Some observations concerning the production of a phagocyte-inhibiting factor *in vitro* are included.

MATERIALS AND METHODS

Strains and suspensions

As in former studies, the strains mostly used were the avirulent Tjiwidej smooth, referred to throughout as TS, and the fully virulent MP6 (Burrows and Bacon, 1954*a*). They were maintained in the dried state. Stock suspensions were prepared from dried material by inoculation into flasks of a semi-synthetic medium, developed by Ross, Hakes and Herbert (1956), which were kept well shaken throughout incubation for 48 hr. at 28°. Population densities of 1–2 $\times 10^{10}$ organisms per ml. were routinely achieved. These stock suspensions were kept at 4° where they retained full viability for at least 6 weeks. Slopes containing 5 ml. of tryptic meat digest agar (TMA) were inoculated with 2 drops of the stock suspension, incubated for 17 hr. at 28°, unless otherwise stated, and the resulting growth suspended in phosphate buffer pH 7·0 to the density required for use. The use of stock suspensions was a deviation from former procedure designed to ensure uniform inoculation of slopes in an endeavour to obtain more uniform suspensions. The stock suspensions were replaced at 4-week intervals.

Suspensions of mouse polymorphonuclear leucocytes

White mice (Porton strain weighing 30–35 g.) were injected intraperitoneally each with 1·5 ml. of 2 per cent wheat starch in physiological saline. After 16 hr. 1 ml. of a solution containing KH_2PO_4 3·6 g., NaCl 3·6 g., sodium acetate 1·6 g., heparin 0·15 g., glucose 4·0 g. and gelatin 10 g. per l., pH 7·0, was injected i.p. into each mouse. This solution was based in part on the recommendations of Tullis (1952) and is referred to as PGA. The animals were killed 5 min. later, by cervical dislocation, and the fluid in the peritoneal cavity containing polymorphs in suspension gently transferred to a paraffin waxed tube, using a waxed pipette. In most experiments the peritoneal fluids derived from ten mice were pooled for use. To this volume of pooled fluid 1 ml. of freshly drawn mouse heart blood was added, the waxed tube sealed with a waxed bung and placed at 4° until required. The presence of erythrocytes in the polymorph suspension facilitated subsequent microscopic examination of stained films by serving to delineate the extent of the spread material so that the edges, where polymorphs accumulated, could easily be found.

From 10 mice *ca.* 7·5 ml. of suspension could be recovered, each 1 ml. containing *ca.* 15 million polymorphs. When necessary the suspension was concentrated by centrifuging at 500 r.p.m. for 5 min. and removing the appropriate volume of supernatant. After harvesting from the mouse or after centrifuging or storage at 4° polymorph suspensions were incubated for 30 min. at 37° to permit recovery from any adverse effects prior to use in a phagocytic system. In our experience mouse polymorphs *in vitro* required the gentlest treatment to avoid their disruption and the composition of the medium for harvesting was critical. The addition of gelatin greatly assisted the preservation of normal morphology and ingestive capacity. Fragility of polymorphs *in vitro* did not appear to result from ascorbic acid deficiency (Nungester and Ames, 1948) or from incompatibility of different pooled peritoneal fluids. Heparin proved a more satisfactory anti-coagulant than sodium citrate or ethylenediamine tetra-acetic acid.

In practically all experiments polymorphs were used within 2 hr. of collection from mice.

Phagocytosis

To compare the abilities of different media to permit the change from sensitivity to resistance to phagocytosis 1 ml. volumes of the media under test were inoculated with 0·1 ml. of suspensions in buffer containing $2·5 \times 10^9$ organisms per ml. The tubes containing the inoculated media were then rotated at 55 r.p.m., with their long axes inclined at 30° to the horizontal, for the required time and at the required temperature. The tubes were then centrifuged at 3000 r.p.m. for 20 min. and supernatants completely removed. To the deposited organisms 0·8 ml. of polymorph suspension was added, the organisms re-suspended therein and 0·2 ml. volumes transferred to each of 3 waxed tubes. These were rotated at 55 r.p.m. at 37° (Robertson and Sia, 1924) for 30 min., then plunged in iced water. Films of material from each tube were prepared, on slides kept at *ca.* 40° on a warm surface, using the normal method for preparing blood films. After fixing and staining by Leishman's method, phagocytosis was estimated in the customary manner by counting the numbers of ingested organisms contained in a random sample of 100 polymorphs and the percentage of polymorphs containing ingested bacteria, for each film. The means of the triplicate determinations of phagocytosis are recorded in the Tables by the two numbers representing these two values.

The following procedure was used to determine the ability of strains to excrete factors having an inhibitory action on mouse polymorphs. Organisms were incubated in particular environments for the desired period then centrifuged at 10,000 r.p.m. for 1 hr. Of the supernatants 1 ml. was used to re-suspend 20 million polymorphs, previously deposited by light centrifugation in waxed tubes, and the mixture incubated at 37° for the prescribed time. The polymorphs were again deposited, the supernatants completely removed, and the polymorphs re-suspended in 1 ml. of PGA. Of these suspensions 0·2 ml. volumes were transferred to waxed tubes, 0·05 ml. of a suspension of the test organism (usually TS) at a density of 5×10^9 organisms per ml. was added to each tube and the tubes rotated and films prepared as above. Fresh suspensions of organisms and of polymorphs were employed for each experiment.

An electrically operated device, which incorporated three Post Office telephone meters, greatly reduced the tedium of phagocytosis estimations.

RESULTS

In experiments *in vitro* the sensitivity to phagocytosis of virulent organisms harvested from TMA slopes which had been incubated at 37° varied very considerably from experiment to experiment. Frequently this sensitivity was lower than was desirable in experiments aiming to determine factors controlling the development of resistance. From slopes which had been incubated at 28° organisms consistently showed high sensitivity and were therefore more satisfactory for

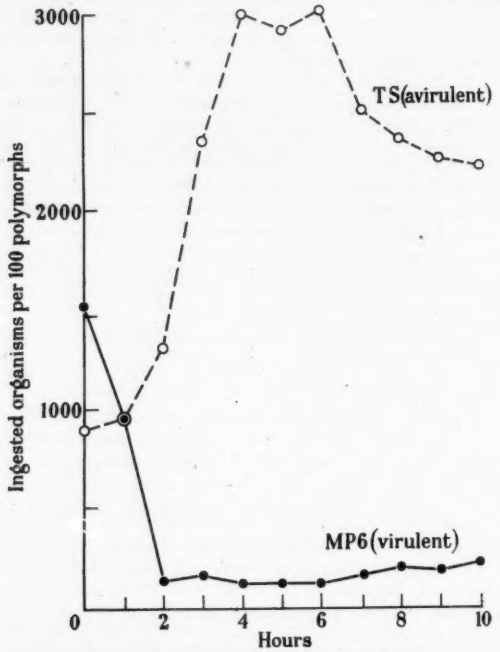

FIG. 1.—Effect of time of incubation on development of resistance to phagocytosis—1 ml. volumes of tryptic digest meat broth pH 7·0 containing $2{\cdot}5 \times 10^8$ virulent or avirulent organisms incubated with rotation at 37° and phagocytosis of resulting organisms determined at hourly intervals.

our purpose. In view of the known temperature dependence of the metabolic processes leading to the accumulation of capsular (or "envelope") material in *P. pestis* (Schütze, 1932), and of the correlation between capsulation and resistance to phagocytosis in other organisms, observations were made of the extent of capsulation of organisms following various treatments leading to resistance. However, no correlation was found between sensitivity to phagocytosis and absence of a visible degree of capsulation with our virulent strain, or between resistance and visible capsulation with our avirulent strain, under our conditions. Although we did not observe visibly capsulated virulent organisms to show high sensitivity, non-capsulated virulent organisms frequently showed a

high level of resistance. These observations suggested that resistance was independent of capsular material.

Preliminary trials clearly showed that sensitive virulent MP6 organisms became resistant to phagocytosis when incubated for 2 hr. at 37° in normal mouse, rabbit or horse serum, and that avirulent TS organisms failed to do so. Similar incubation of these two representative strains in certain batches of tryptic meat broth (TMB) also resulted in the development of resistance by MP6 and in the retention of sensitivity by TS. Our earlier experiments were devoted to

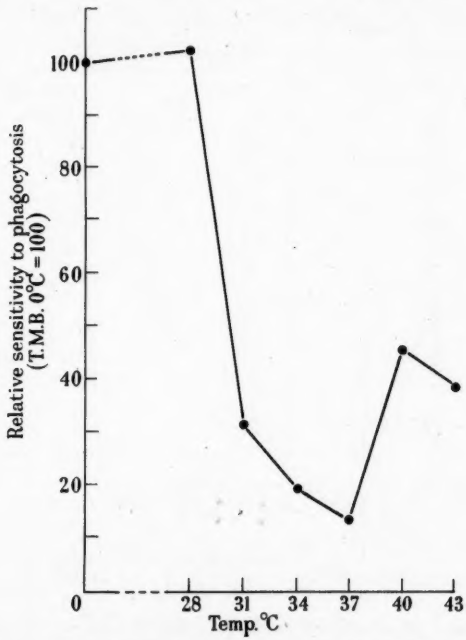

FIG. 2.—Effect of temperature on development of resistance to phagocytosis—1 ml. volumes of tryptic digest meat broth pH 7·0 containing $2{\cdot}5 \times 10^8$ virulent organisms incubated for 3 hr., with rotation, at temperatures shown and phagocytosis of resulting organisms determined.

establishing, so far as possible, the optimum conditions for the development of resistance by virulent organisms in this readily available medium. Maximum resistance was found to result within 3 hr. of incubation, with rotation, at 37° in TMB having an initial pH within the range 6·2 to 7·4. Resistance did not develop within this period in static culture, and developed less in shaken culture than when gentle rotation was employed. Fig. 1, 2 and 3 illustrate the effects of varying the time and temperature of incubation and the initial pH of TMB individually while keeping the other important factors at optimal values.

Complete resistance to phagocytosis was never achieved in these experiments. We think it probable that organisms remaining sensitive after the bulk of the bacterial population has developed resistance constitute the non-viable fraction

of the population. It can be seen from Fig. 1 that even following prolonged periods of incubation in TMB this residuum of sensitive organisms remains.

The very different behaviour of avirulent TS organisms under the conditions which permit virulent MP6 organisms to develop maximum resistance to phagocytosis is illustrated in Fig. 1. As judged by the method employing India ink for capsule demonstration (Rowland, 1914), neither strain showed visible capsulation of organisms over the period 0–3 hr. in the experiment from which the results given in Fig. 1 were taken. Visible capsulation was first evident with both strains

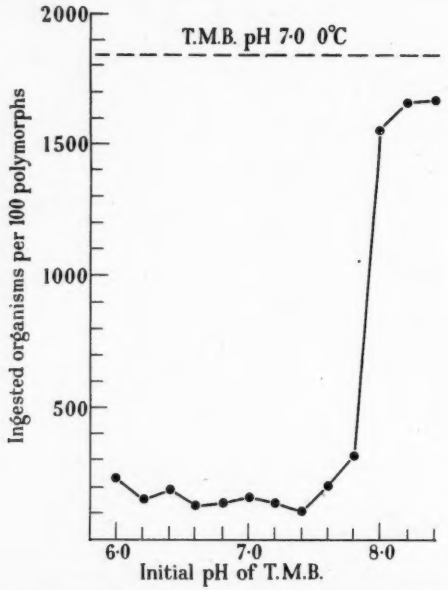

FIG. 3.—Effect of initial pH of medium on development of resistance to phagocytosis—1 ml. volumes of tryptic meat digest broth, previously adjusted to the pH values shown, inoculated with $2{\cdot}5 \times 10^8$ sensitive virulent organisms and incubated with rotation for 3 hr. at 37°. Phagocytosis of resulting organisms then determined.

at 5 hr., that is at a time well after maximum resistance of virulent organisms had been reached, and was very evident in both strains by the 10th hr. In this well capsulated state TS retained high sensitivity to phagocytosis. We concluded from these observations that resistance to phagocytosis was not determined exclusively by the presence of a degree of capsulation observable by the India ink method.

Both virulent and avirulent organisms multiplied slowly when incubated with rotation in TMB pH 7·0 at 37°. With 3 hr. incubation both types increased their viable count by a factor of *ca.* 1·8 only.

The test for ability to develop resistance, or failure to do so, when incubated under precise conditions in TMB readily allowed us to differentiate our virulent and avirulent representative strains MP6 and TS. This test was applied to a

random selection of laboratory strains of *P. pestis* to confirm the generality, or otherwise, of this distinction between virulent and avirulent types. Organisms derived from the same suspensions as those used for the estimations of sensitivity to phagocytosis were injected intraperitoneally into mice, at levels of 10^4 organisms per mouse, a dose which was known from experience to differentiate clearly virulent from avirulent strains. The results are given in Table I.

TABLE I.—*Differentiation of Virulent from Avirulent Strains of* Pasteurella pestis *by Phagocytosis* In Vitro.

Experiment.	Strain.	Phagocytosis.*	Assessment by phagocytosis *in vitro*.	Virulence† for mice.
1	MP6	30 : 20	V	10
	TS .	1522 : 99	AV	0
	A1122	1567 : 97	AV	0
	5341	1159 : 98	AV	0
	14 .	1842 : 98	AV	0
	I–72	119 : 52	V	8
	F9581	55 : 25	V	10
	L27	40 : 23	V	10
	L36	47 : 20	V	10
2	MP6	41 : 20	V	10
	TS .	1541 : 94	AV	0
	Java	1883 : 100	AV	0
	Soemedang	1422 : 100	AV	0
	A2256 Ppl	1203 : 98	AV	0
	H327	49 : 21	V	10
	139L	43 : 19	V	10
	Yokohama	22 : 13	V	10
	M1 (purine dependent)	51 : 22	V	0

Organisms harvested from 18-hr. growths on tryptic digest meat agar at 28°, incubated with rotation for 3 hr. at 37° in tryptic digest meat broth pH 7·0 ($2{\cdot}5 \times 10^8$ organisms/ml.) and tested for sensitivity to phagocytosis *in vitro*.

* Phagocytosis recorded by numbers of organisms ingested by 100 polymorphs, followed by percentage of polymorphs containing ingested organisms.

† Virulence recorded by deaths out of 10 mice injected intraperitoneally with 10^4 organisms suspended in 0·2 ml. buffer.

V = virulent. AV = avirulent.

With the exception of strain M1, the assessments of virulence or of avirulence on the basis of this test *in vitro* agreed completely with the assessments based on animal assay. Strain M1 was derived from MP6 and is characterised by dependence on exogenously supplied purine for growth (Burrows and Bacon, 1954*b*). Its avirulence reflects the non-availability of purines in the mouse. In the presence of purines, *e.g.*, in TMB, this mutant shows all the properties of its virulent parent, including the ability to develop resistance to phagocytosis.

From time to time different batches of TMB either failed to permit virulent organisms to develop resistance or permitted the development of moderate resistance only. Certain of these batches were improved by the addition of glucose and/or cystine but others were not. These failures stimulated an attempt to define the minimal nutritional conditions necessary to permit the development of resistance by virulent organisms in synthetic media. Despite exhaustive trials no entirely satisfactory chemically defined medium was evolved which would

regularly permit MP6, previously grown at 28°, to develop a level of resistance comparable with that achieved in effective batches of TMB. A medium containing phosphate buffer, glucose, acetate, asparagine, cystine, methionine, phenylalanine, valine, leucine, isoleucine, proline, glutamic acid, spermine, haemin, biotin, pantothenate and magnesium salts, pH 7·0, at times permitted an impressive development of resistance, yet at other times, under what we believed to be identical conditions, failed to be effective.

As mentioned earlier, virulent organisms derived from TMA slopes incubated for 17 hr. at 37° showed widely varying degrees of resistance from day to day. This variation ranged from the high sensitivity of organisms recovered from growths at 28° to the high resistance achieved by such organisms following 3 hr. incubation in TMB at 37°. Organisms recovered in a sensitive condition from slopes grown at 37° rapidly became resistant on incubation in TMB at 37° and frequently, but not invariably, on incubation in phosphate buffer supplemented with ammonium salts, glucose and cystine pH 7·0 at 37°. The high sensitivity of organisms recovered from slopes incubated at 37° apparently resulted from previous exhaustion of glucose, or its equivalent, during the 17 hr. incubation period. Supplementation of these exhausted slopes with buffer plus glucose, followed by a further 2 hr. incubation at 37°, yielded organisms with considerably increased resistance. Supplementation with buffer alone was ineffective.

Growth of virulent organisms in TMB at 37° to population densities in excess of *ca.* 2×10^9 organisms per ml. yielded organisms with low resistance to phagocytosis. Supplementation of TMB with galactose (a sugar whose metabolism by *P. pestis*, unlike that of glucose, did not result in gross alteration of the pH of the medium) permitted the recovery of larger populations showing a high level of resistance. Fig. 4 illustrates this effect.

Virulent organisms did not achieve high resistance following incubation in CCY medium (Gladstone and Fildes, 1940) at pH 7·0 for 3 hr. at 37°. The efficiency of this medium was however considerably improved by the addition of glucose as illustrated in Table II.

TABLE II.—*Effect of Added Glucose on Development of Resistance to Phagocytosis by Virulent Organisms Incubated in CCY Medium for* 3 *hr.*

Medium.	Addition.	Temperature.	Phagocytosis.*
CCY	—	0°	1365 : 87
,,	—	37°	589 : 82
,,	Glucose 0·1%	,,	181 : 58
,,	,, 0·5%	,,	184 : 62
,,	,, 1·0%	,,	127 : 50
,,	,, 1·0% + cystine 200 μM	,,	103 : 47
,,	Cystine 200 μM	,,	895 : 92
TMB	—	,,	76 : 33

* Phagocytosis recorded by numbers of organisms ingested per 100 polymorphs, followed by per cent of polymorphs containing ingested organisms.

These observations are consistent with the view that the development of resistance is in part dependent on the free availability of a utilisable carbohydrate and that metabolism in the absence of free carbohydrate results in failure to develop resistance, or in loss of resistance previously acquired.

Persistence of the resistant state

As shown in Fig. 1, virulent organisms after acquiring resistance retain the property for at least a period of 7 hr. of continued incubation in TMB at 37°. When rendered resistant by incubation in TMB and stored in the refrigerator they retained resistance for 4 to 5 hr. and then rapidly became sensitive. Low

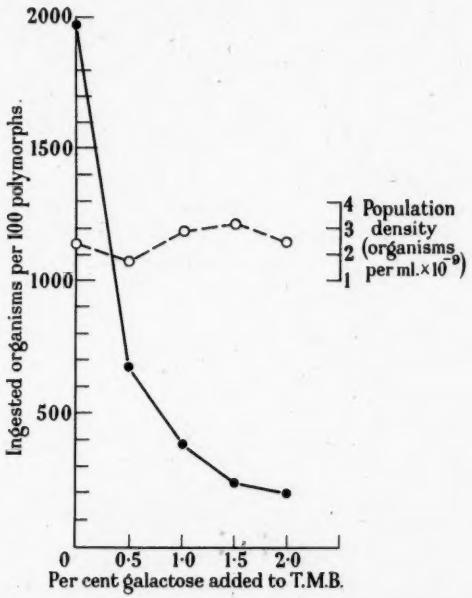

FIG. 4.—Effect of galactose on resistance to phagocytosis of large populations of virulent organisms. Tryptic digest meat broth pH 7·0 supplemented with galactose to the concentrations shown and inoculated with sensitive virulent organisms to a density of 2×10^8 organisms per ml. Incubated static for 11 hr. at 20°, followed by 6 hr. at 37° rotated. Volumes containing equal numbers of organisms centrifuged and organisms tested for resistance to phagocytosis.

concentrations of streptomycin (10μg./ml. final conc.) added to TMB completely suppressed the development of resistance by sensitive virulent organisms. However high levels of streptomycin (400 μg./ml. final conc.) acting on resistant organisms for 1 hr. at 37° did not render them sensitive to phagocytosis. Similarly, treatment for 30 min. at 37° in M/10 KCNS—a reagent whose action results in solution of capsular material (Amies, 1951)—or with formalin or tannic acid, both at 0·5 per cent final conc., followed by two washings in saline, did not materially affect the resistance of virulent organisms. Treatments in saline followed by two washings in saline were used as controls. Thus neither agents acting as metabolism inhibitors, nor those which would be expected to react non-specifically with surface proteins, materially affected the resistance of virulent organisms once they had achieved a resistant state.

Production of polymorph-inhibiting factor(s) in vitro

Concurrently with the development of resistance by virulent organisms *in vivo* there is evidence that the phagocytic activity of polymorphs in infected mice becomes seriously impaired, rendering them incapable of ingesting a secondary infection with phagocytosis-sensitive avirulent organisms (Burrows and Bacon, 1954*b*). However *in vitro* the development of resistance by virulent organisms in TMB was not accompanied by the excretion into the medium of detectable amounts of a factor, or factors, which inhibited the ingestive capacity of mouse polymorphs. Similarly we failed to demonstrate the production of a free polymorph-inhibiting factor during the development of resistance by virulent organisms incubated in horse serum, under a number of conditions. These conditions included variation of O_2 and CO_2 tensions.

Successful demonstrations of the production of a polymorph-inhibiting factor *in vitro* have, to date, been confined to experiments in which mouse peritoneal

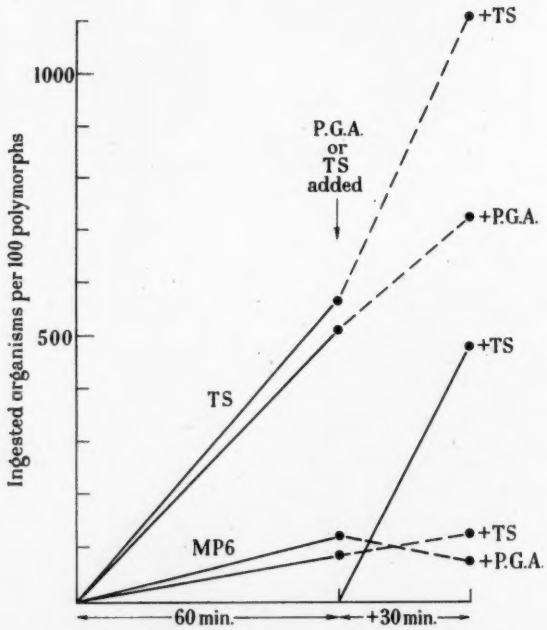

FIG. 5.—Inhibition of the phagocytic activity of mouse polymorphonuclear leucocytes following incubation with virulent organisms. Virulent MP6 and avirulent TS organisms grown at 28° then incubated with rotation 3 hr. at 37° in tryptic digest meat broth pH 7·0, sedimented and re-suspended in PGA to 1×10^9 organisms per ml. Polymorph suspension in PGA plus mouse peritoneal fluid distributed in 0·2 ml. volumes to 15 waxed tubes. To 3 was added 0·025 ml. PGA, to six 0·025 ml. TS suspension and to remaining six 0·025 ml. MP6 suspension. After rotation at 37° for 1 hr. and preparing films from each tube 0·025 ml. TS suspension was added to the three tubes originally receiving PGA, and to three of each of six tubes originally receiving TS or MP6 suspension. The remaining 3 of each series of 6 received 0·025 ml. PGA. All tubes then rotated at 37° for further 30 min. and films prepared from each and phagocytosis estimated.

fluid or heparinised normal mouse whole blood have been present in media in which virulent organisms were incubated at 37°.

An indirect demonstration of the production of inhibition by virulent organisms, and not by identically treated avirulent organisms, is given in Fig. 5. The experiment compared the ability to ingest TS organisms of polymorphs exposed to the products of virulent or of avirulent organisms, in a phagocytic system containing mouse peritoneal fluid.

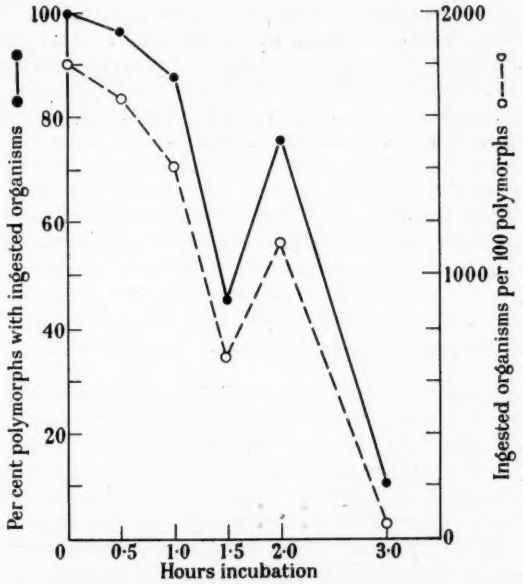

FIG. 6.—Production of polymorph-inhibiting factor(s) by virulent organisms incubated in mouse whole blood. Heparinised (1/6500 heparin final conc.) normal mouse whole blood inoculated with virulent organisms to 1×10^9 per ml. and incubated at 37° with rotation for times shown. Cultures centrifuged and 0·8 ml. supernatants added to 0·2 ml. of a suspension containing 1×10^8 polymorphs per ml. in PGA in waxed tubes. Tubes were then rotated for 2 hr. at 37° From each tube three 0·2 ml. volumes transferred to waxed tubes, 0·05 ml. of a suspension of TS (5×10^9/ml.) added to each; rotated 30 min. 37° films prepared and phagocytosis estimated.

It is evident that polymorphs incubated with resistant virulent organisms have their ability subsequently to ingest sensitive organisms very greatly reduced.

Fig. 6 gives the results of an experiment to estimate the effect of time of incubation on the production of a polymorph-inhibiting factor by virulent organisms incubated at 37° in heparinised whole mouse blood. The magnitude of this production varied in three experiments of this type but the peculiar inflection of the curve at the two-hour plot was a constant feature. Its significance is not understood.

We have no evidence that avirulent organisms at any time excrete a polymorph-inhibiting factor *in vivo* or *in vitro*. In contrast to peritoneal smears

derived from mice moribund from virulent infection, those from mice moribund from infection with avirulent organisms (2×10^8 per mouse) show the majority of polymorphs to contain large numbers of ingested organisms. It is unlikely that such ingestion would be observed if the large numbers of uningested organisms present were excreting, or had excreted, effective amounts of a polymorph-inhibiting factor.

Destruction of P. pestis *following phagocytosis*

Information as to whether or not phagocytosis of virulent organisms leads to their destruction was considered of importance in this study. Numerous observations of stained preparations during estimations of phagocytosis allow us to state that both ingested virulent and avirulent organisms stained less densely than did uningested organisms and, with further time, became rounded and swollen and eventually unrecognisable. Again, in experiments *in vivo*, the high ingestion of sensitive virulent organisms observed immediately following injection progressively declined to become practically zero within a 5-hr. period (Burrows and Bacon, 1954*b*). If ingested organisms remained stainable and recognisable this decline would not have been observed, unless, during this period, a complete replacement of the polymorphs originally present by fresh elements had occurred. In closed phagocytic systems *in vitro*, where such replacement cannot occur, a decline in phagocytosis with time was repeatedly observed. Indirect evidence therefore is in support of the destruction of organisms following phagocytosis.

As a direct approach to this problem the following experiments were conducted. Sensitive virulent organisms were added to a suspension containing known numbers of polymorphs and phagocytosis allowed to proceed. The polymorphs were sedimented and washed twice with PGA and numbers of ingested organisms determined on a small sample. The remainder were re-suspended in PGA to known concentration and divided into two parts. To one was added normal rabbit serum, to the other an equal volume of an antiserum prepared in rabbits against mouse polymorphs, and both incubated 1 hr. at 37°. This antiserum caused the lysis of mouse polymorphs with the liberation of their contents but did not affect the viability of virulent organisms.

Equal volumes of the two suspensions were then plated on TMA containing sodium metabisulphite at 0·075 per cent final conc. and incubated for 48 hr. at 37°. This medium was effective for the growth of *P. pestis* to colony size from single organisms. If organisms remained viable after ingestion each polymorph containing one or more ingested organisms should have given rise to one colony (normal serum treated) and each ingested organism liberated by antiserum treatment similarly should have produced one colony. The results are given in Table III.

In other experiments the lethal effects on mice of known numbers of virulent organisms, contained intracellularly in mouse polymorphs, were compared with those of extracellular organisms. To sterilise organisms adhering to polymorph surfaces the polymorphs containing ingested organisms were incubated with streptomycin, an antibiotic to which the polymorph cell membrane apparently is impermeable (Schaffer, Kucera and Spink, 1953). The results indicated that for lethal effects equivalent to those of 150 extracellular organisms, or to 150 extracellular organisms surviving streptomycin treatment, some 26,000 ingested

TABLE III.—*Destruction of Virulent Organisms by Phagocytosis*

		Expt. 1.*	Expt. 2.†
Normal serum treated	Number of colonies expected if each polymorph with ingested organisms contains at least one viable organism	114	152
	Colonies obtained	58	92
Lytic serum treated	Number of colonies expected if all ingested organisms were liberated and viable	1631	3488
	Colonies obtained	77	52

* Expt. 1.—983 organisms ingested per 100 polymorphs ; 69 per cent polymorphs contained ingested organisms.

† Expt. 2.—2089 organisms ingested per 100 polymorphs ; 91 per cent polymorphs contained ingested organisms.

Polymorph suspensions diluted after treatment so that plated volumes contained 166 whole or disrupted polymorphs.

virulent cells were necessary. These results support the conclusion that phagocytosed virulent organisms are rendered non-viable.

DISCUSSION

The progress of this work has been hampered by the difficulty of obtaining reproducible experimental results from day to day. In addition, experiments which in the past were readily confirmed, have, when repeated many months later, failed to yield results in accordance with earlier findings. As a result much experimental work has had to be omitted from this paper. The behaviour of *P. pestis in vitro* appears to be greatly influenced by subtle changes in the environment which at present we cannot completely define. As an example, when the work was started we were able consistently to convert sensitive virulent organisms, taken from growths at 28°, to the resistant state by incubation at 37° in a medium containing phosphate, ammonium salts, glucose and cystine. This effect was sufficiently reproducible to permit titration of optimal levels of cystine for this development. Maximum resistance developed in 3 hr. over the range 25–400 μM cystine ; levels between 400–1600 μM were less effective, and above 1600 μM was inhibitory. The same experiments repeated a year later failed to yield resistant organisms at any cystine level. The possibility of genetic variation of our organisms maintained in the dried state cannot be excluded, neither can the possible selection of different substrains during the growth of stock suspensions. These latter however were all routinely checked for virulence for mice, and for other properties before being brought into use, but variations in virulence or other properties were not detected.

The ability of virulent organisms to develop resistance to phagocytosis under defined conditions *in vitro*, and under the conditions existing *in vivo* clearly distinguishes them from avirulent laboratory strains of *P. pestis*. The suppression of the development of resistance by streptomycin indicates that active metabolism of the virulent organism is essential for this process. This supports the conclusions from earlier studies on the behaviour of the purine-dependent mutant M1 *in vivo*. Further, metabolism in the presence of an available carbo-

hydrate at temperatures close to 37° are necessary conditions for the expression of the potentiality for the development of resistance.

Present evidence does not permit us to decide whether or not the factor determining resistance to phagocytosis is identical with that causing impairment of the phagocytic activity of mouse polymorphonuclear cells. While resistance can be achieved in a variety of media, a free phagocytosis-inhibiting factor has only been demonstrated in media containing mouse fluids. This suggests that resistance and inhibition are independent phenomena. However, if we assume that an excreted factor protects resistant organisms from phagocytosis, and if in sufficient quantity can impair the ability of polymorphs to phagocytose sensitive organisms, our findings could be explained on the basis of the differing quantitative production of this factor in media with and without added mouse fluids; or on equal production in both but with instability in the absence of the latter. Provided a sufficiency of the factor was produced to give maximum resistance, amounts of the factor in excess would make no contribution in our tests for resistance, but would be detectable in a test for inhibition of phagocytosis. We are now developing new techniques which may permit a decision to be made as to the identity or otherwise of resistance factor(s) and polymorph-inhibiting factor(s).

Our experiments similarly do not give unequivocal evidence supporting the view that virulent organisms in the sensitive and resistant conditions differ in their surface properties rather than in their excreted soluble metabolic products. Neither do they tell us whether the important differences between resistant virulent and avirulent organisms, which can be detected by the tests described in this paper, are associated with antigenic differences hitherto not recognised. Studies which indicate that virulent organisms in the resistant condition do in fact possess an antigen not evident in sensitive virulent, or avirulent, organisms will be reported later.

SUMMARY

Virulent strains of *Pasteurella pestis* can be differentiated from protective avirulent strains by phagocytic tests *in vitro*. When grown on agar slopes at 28° both strains are highly sensitive to phagocytosis by mouse polymorphonuclear leucocytes. Virulent organisms grown at 28° and then incubated for 3 hr. in tryptic digest meat broth at 37° change to a condition in which they are highly resistant to phagocytosis. Avirulent organisms treated identically retain their high sensitivity to phagocytosis. The degree of aeration, pH of the medium, time and temperature of incubation and availability of a carbohydrate source are factors determining the suitability of media to permit the change of virulent organisms from the sensitive to the resistant state. In media containing mouse peritoneal fluid or mouse whole blood virulent organisms produce a factor(s) which inhibits the phagocytic activity of mouse polymorphonuclear leucocytes. Evidence is given that phagocytosed virulent organisms are rendered non-viable.

We gratefully acknowledge the interest and advice of Dr. D. W. Henderson throughout this work. We also wish to express our appreciation of the excellent technical assistance rendered by Miss Barbara Alkins, Mrs. Corinne Ness and Miss Dorothy Burrell.

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A STUDY OF THE IMMUNITY AGAINST TETANUS TOXIN FOLLOWING THE USE OF A COMBINED TYPHOID-TETANUS VACCINE

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COMBINED prophylactics are being more and more extensively used and problems in connection with their use are being studied in increasing detail. One of the most controversial questions is whether the immune response to each component of a combination is equivalent to that given when the component in question is the sole antigen. Although the overwhelming majority of authors (Ramon and Zoeller, 1926; Ando and Nagata, 1935; Faragó, 1938; Maclean and Holt, 1940; Rogozin, 1940; Bigler and Werner, 1941; Regamey, 1948; Truhmanov and Pafnuteva, 1955; Truhmanov and Krasznova, 1955) report beneficial effects from a variety of combined vaccines, and claim that the single components in the combined prophylactic were just as, or even more, effective than when used alone, from time to time reports appear in the literature on the antagonistic action of antigens injected simultaneously or at different times. For example, Michaelis (1904) reported that the globulin fraction of serum " crowded out " the action of the albumin fraction antigen and suggested the term " antigenic competition " to denote the phenomenon. In his rabbit experiments Friedberger (1904) found that combined typhoid-cholera vaccine produced much less cholera agglutinins than did the pure cholera vaccine. Benjamin and Witzinger (1912) demonstrated that a previous horse serum injection prevented the formation of sheep erythrocyte haemolysins. Glenny, Hopkins and Waddington (1925) showed that rabbits immunised with animal serum responded to a subsequent injection of diphtheria antigen with a very low antitoxin production. Zlatogorov, Glusman and Kandyba (1929) found that guinea-pigs gave a decreased response to a combined diphtheria-tetanus vaccine, as compared to that given when the antigens were injected one by one. In recent years, Barr and Llewellyn-Jones (1953*a*, *b*; 1955) studied the " competition of antigens " in detail and found that animals immunised previously with a single antigen responded to a subsequent immunisation with a combined vaccine with a production of antibodies to the newly introduced component which was much lower than that in the controls.

The above outlined animal experiments have shed light on the theoretical significance of the problem, but the results obtained are, of course, not applicable without restrictions to man. Thus it is very important to decide whether in practice, in compulsory vaccinations carried out on a large scale, the competition of antigens would not have an untoward effect on the results. The final solution will have to wait, until large numbers of detailed studies have been carried out, since the results are highly dependent on the quality of the antigen used, on the interval between single inoculations and on other factors. Here, we wish to find the answer to the

question : Is the tetanus component in a combined typhoid-tetanus prophylactic not less effective in individuals previously immunised against typhoid ?

EXPERIMENTAL

In order to elucidate this point, 956 children living in Budapest and Székesfehérvár were inoculated twice at 7-week intervals with a combined typhoid-tetanus vaccine (basic immunisation). This work was carried out within the framework of the compulsory inoculation of 12 years-old schoolchildren in April and May 1954. The Budapest schoolchildren had been given no anti-typhoid vaccine before, while those living in Székesfehérvár had been vaccinated against typhoid on three occasions : in 1950, in 1952 and in 1953. None of the groups had been given inoculation against tetanus. The boosting inoculations were given 9 months after the basic immunisation, in February 1955, injecting the combined typhoid-tetanus prophylactic. Of the 956 children 823 were given the boosting inoculation. The combined typhoid-tetanus vaccine produced by the " Human Institute for the Production of and Research on Vaccines " was used throughout (preparation No. 2/17 in the basic, and No. 11/4 in the boosting inoculations). This vaccine contains the trichloracetic acid extract of typhoid bacteria, and tetanus anatoxin, purified with trichloracetic acid and adsorbed on aluminium hydroxide gel (in 1 ml. there was typhoid extract equivalent to 1000 million bacilli and tetanus anatoxin of a binding power of 5). Each inoculum was 1 ml. in volume. The injection was given deep subcutaneously on the extensor aspect of the left arm.

Blood samples taken at 2 weeks and 9 months after the basic immunisation, and at 2 weeks and 7 months after the boosting inoculation were tested for tetanus antitoxin titre, in mice weighing 14–16 g., at a level of L+/400. A standard titrated against the Copenhagen one has been used as the control. The single A.U. levels were indicated by the death of the animals at 5 days.

At 2 weeks following basic immunisation the sera were tested in the following way : Aliquots of 20 sera were pooled and titrated precisely. A total of 420 sera was examined by this method. Each serum was tested individually, too, but only for the presence or absence of 0·1 A.U./ml.

Later each serum was titrated individually.

RESULTS

The antitoxin titres for sera taken at 2 weeks following the basic immunisation were determined first and are presented in Table I. At 2 weeks following basic

TABLE I.—*Tetanus Antitoxin Titres at 2 Weeks following the Basic Immunisation*

	Budapest.	Székesfehérvár.
Values for pooled sera		
(a) number of sera	280	140
(b) average titre A.U./ml.	0·9	0·8
Individual titres, A.U./ml.		
0·1 and higher	405	212
< 0·1	10	4
Total	415	216

We are indebted to Dr. G. Barsy for the calculation of the average values presented in this Table and for the final construction of the Tables.

immunisation with the combined typhoid-tetanus vaccine comparable tetanus antitoxin titres were obtained in the individuals not immunised against typhoid before and in the individuals repeatedly immunised against typhoid. This applies to pooled sera, in the first place, but the results of individual titrations were not much different, either, inasmuch as the frequency of sera showing titres lower than 0·1 A.U./ml. was comparable in the two groups (2 and 2·5 per cent respectively).

Thus, at 2 weeks following the basic immunisation the two groups gave closely similar immune responses. After that, sera obtained at 9 months following the basic immunisation were examined for antibody titre in order to determine whether there were differences in the persistence of antibodies between the two groups. The results are summarised in Table II. It can be seen that at 9 months

TABLE II.—*Tetanus Antitoxin Titres at 9 Months following the Basic Immunisation*

	Individual titres, A.U./ml.			
	<0·01	0·01–0·1	0·1–1	Total
Numbers :				
Budapest . . .	10	189	21	220
Székesfehérvár . .	5	87	11	103
Percentages :				
Budapest . . .	4	86	10	100
Székesfehérvár . .	5	84	11	100

following immunisation with the typhoid-tetanus vaccine there was no difference in the level of tetanus antitoxin between the two groups, just as no such difference could be detected at 2 weeks following basic immunisation.

The basic immunisation serves only to prepare the system for the boosting inoculation. The efficacy of immunisation will be ultimately determined by the ability or failure of the system given the boosting inoculation to produce in time sufficient quantity of antibodies in an emergency (for example, on sustaining injury) ; in other words : Is the potential immunity of the individual adequate ? To elucidate this point, sera obtained at 2 weeks following the boosting inoculation from both groups were tested for tetanus antitoxin titres. The results are summarised in Table III.

TABLE III.—*Tetanus Antitoxin Titres at 2 Weeks following the Boosting Inoculation*

	Individual titres, A.U./ml.				
Designation.	<0·1.	0·1–1·0.	1·0–10·0.	>10·0.	Total.
Numbers :					
Budapest . .	3	36	123	18	180
Székesfehérvár .	—	24	102	15	141
Percentages :					
Budapest . .	2	20	68	10	100
Székesfehérvár .	—	17	72	11	100

It will be seen that at 2 weeks following the boosting inoculation the antibodies to tetanus rose to equal levels in both groups. The percentage deviations are negligible.

Since it is the measure of the potential immunity that decides the effectiveness of immunisation, it was deemed necessary to analyse the results in more detail ; the tenfold steps used may have masked eventual finer differences. For this reason, an analysis was made of the intervals after inoculation at which mice succumbed between the single A.U. levels. The percentage mortality rates for the two groups were almost identical within the single antitoxin levels at 2, 3, 4 and 5 days alike,

i.e., no differences in tetanus antitoxin titres could be demonstrated between the two groups in this way, either.

For the sake of completeness, sera taken at 7 months following the boosting inoculation were also tested for tetanus antitoxin titres. The results are presented in Table IV.

TABLE IV.—*Tetanus Antitoxin Titres 7 Months following the Boosting Inoculation*

Titres, A.U./ml.	Budapest.	Székesfehérvár.
<0·01	1	1
0·01–0·1	13	10
0·1–1·0	48	64
1·0–10·0	29	21
Total	91	96

These show that at 7 months following the boosting inoculation the individuals inoculated with the combined typhoid-tetanus prophylactic showed equal levels of tetanus antitoxin, irrespective of their having been inoculated against typhoid prior to the basic immunisation or not. It is to be noted that the two groups were so selected that the individuals giving equal titres at 2 weeks following the boosting injection are represented in equal percentage in the two groups. Thus, in conclusion it can be stated that the individuals given no previous inoculation against typhoid and those inoculated several times responded to the combined typhoid-tetanus prophylactic with equal tetanus antitoxin production, as determined at different points of time.

Since the results for the two groups were closely similar, they could be merged so as to obtain a general view of the effectiveness of the combined prophylactic in large material. The summarised data are presented in Table V. Sera from

TABLE V.—*Tetanus Antitoxin Titres at Different Intervals After Immunisation (Tests on 1251 Sera)*

	Following basic immunisation.		Following boosting inoculation.	
Titres, A.U./ml.	2 weeks.	9 months.	2 weeks.	7 months.
<0·01	—	15	—	2
0·01–0·1	—	276	3*	23
0·1–1·0	380	32	60	112
1·0–10·0	40	—	225	50
>10·0	—	—	33	—
Total	420	323	321	187
Average titre	0·87†	0·04	2·50	0·42

* Below 0·1 A.U./ml. † Average titre of pooled sera.

children inoculated twice at an interval of 7 weeks contained at 2 weeks following the second inoculation an average of 1 A.U./ml. In 9 months the average titre fell to about $\frac{1}{20}$ of that; on the other hand, the boosting inoculation raised the titre to about 60 times the original, which fell to about $\frac{1}{6}$ of the latter value in 7 months. It can also be seen that the average titre at 2 weeks following the boosting inoculation was about 3 times higher than that at the same interval after the basic

immunisation. The excellent effect of the boosting inoculation is illustrated even more clearly by the fact that 7 months after the boosting inoculation the average tetanus antitoxin titre was 10 times higher than at 9 months following the basic immunisation.

The children showing low antitoxin titres merit special attention. According to the data in Table V, 9 months after basic immunisation 15 children had titres lower than 0·01 A.U./ml., and at 2 weeks following the boosting inoculation sera from 3 children showed titres lower than 0·1 A.U./ml. Of the 18 children just referred to, 15 could be tested at 7 months following the boosting inoculation and of the latter only 2 showed titres lower than 0·01 A.U./ml.

These two children were then tested in detail. By the D.L.M. neutralisation test it was shown that the sera from the two children neutralised only a minimum amount of toxin (1 to 2 D.L.M.). At 9 months following the boosting inoculation both children were given another boosting inoculation. At five days after that, the serum from one of these children showed an antitoxin titre of 0·1 to 1·0 A.U./ml. which rose to 10 A.U./ml. at 12 days. Sera from the other child were tested at 4 and at 8 days following the second boosting inoculation and were found to show titres lower than 0·01 A.U./ml. on each occasion. Unfortunately, due to conditions beyond our control, no further tests could be carried out.

DISCUSSION

We have sought experimental evidence to determine whether the tetanus component of the combined typhoid-tetanus prophylactic compulsory in Hungary did not lose some of its effectiveness when children inoculated with the combined vaccine at the age of 12 years had been given repeated injections of anti-typhoid vaccine in previous years. It was first of all the animal experiments by Barr and Llewellyn-Jones (1953*a*, *b* ; 1955) that induced us to study this practical problem. We have obtained a negative answer, since, as determined by tests made at different points of time, the individuals inoculated against typhoid before, as well as those never inoculated before showed identical titres of tetanus antitoxin.

We do not wish to imply that under experimental conditions different from those we employed a previous antigenic stimulus might not influence the effectiveness of the heterologous component of a subsequently injected combined prophylactic. The results obtained are dependent on the conditions of the experiment ; for example, on the interval between single inoculations, on the dose of antigen, on the number of inoculations, as well as on the quality of the antigen used. Tomcsik (1939) pointed out the importance of the time factor. According to the guinea-pig experiments of Barr and Llewellyn-Jones (1955) the interval between single inoculations has a significant effect on the effectiveness of combined inoculations. According to Ormay (unpublished findings) the intensity of inhibition is highly dependent on the number of secondary antigenic stimuli. Alatürceva (1953) reported that the response to combined vaccines varied, depending on the nature of the antigens used.

Finally we would like to make it perfectly clear that most of the published work dealing with " interference-effects " applies to the use of two or more protein antigens or where one is a bacterial vaccine. On the other hand, we used for injection such a combined vaccine, of which the typhoid component was a preparation of the Boivin-Mesrobeanu type, that is a carbohydrate-lipid-polypeptide complex. The tetanus component was a simple protein.

After having decided the concrete problem raised, we wish to appraise the effectiveness of the tetanus component of the precipitated typhoid-tetanus vaccine used in Hungary, this time without taking into account any previous inoculation against typhoid. According to Scheibel and Bojlen (1948) the individual immunised actively against tetanus may be considered to be protected if the serum contains at least 0·01—0·02 A.U. per ml. Among the large number of individuals tested by us there were only two showing a titre lower than 0·01 A.U./ml. at 7 months following the boosting inoculation. Tests made after another boosting inoculation have shown that one of the two individuals had a satisfactory potential immunity.

Thus the evidence obtained does not seem to suggest that the typhoid component of the combined typhoid-tetanus prophylactic used by us would lessen the effectiveness of the tetanus component. Further support to our view is lent by the investigations of Erdös (unpublished), who has obtained results closely similar to ours in 46 children of 12 years, inoculated with a precipitated tetanus prophylactic under conditions almost identical with those employed by us.

SUMMARY

In tests made on 956 children, 12 years of age, it has been shown that previous repeated inoculations against typhoid had no unfavourable influence on the effectiveness of the tetanus component in a combined typhoid-tetanus prophylactic, as determined at various intervals following immunisation.

The tetanus component in the combined typhoid-tetanus prophylactic used for deciding this question was of adequate efficacy.

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THE LONG TERM SURVIVAL AND REACTION TO METHYL-THIOURACIL OF THYROID AUTO-IMPLANTS IN THE RAT

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WE record in this paper an attempt to find out whether thyroid auto-implants survive more than a year in hemithyroidectomised rats, whether they survive serial re-implantation and whether they show maintained hyperplasia and ultimate tumour formation in response to prolonged goitrogen treatment. Previous work has been mainly concerned with the early fate of implants (Loeb, 1930). These undergo massive ischaemic coagulation necrosis. Regeneration takes place from peripheral surviving follicles which are revascularised by the surrounding host tissue. A new small nodule of thyroid tissue is established within 2 to 3 weeks. One month after hemithyroidectomy and auto-implantation of one lobe, the residual gland in the neck takes up about 7 times more of a tracer dose of radioactive iodine than the implant (Dempster and Doniach, 1955). Since the implant appears to play no essential part in the body's economy one must consider the unlikely possibility that in time it might wither away. Though the established implant is responsive to a raised or lowered level of circulating pituitary thyrotrophic hormone (T.S.H.), Dempster and Doniach (1955) found that the initial regenerative growth is not abolished by thyroxine treatment and thus appears to be independent of T.S.H. This contrasts with the hyperplasia produced by goitrogens and the compensatory hypertrophy which follows partial thyroidectomy, both of which are abolished by thyroxine administration or by hypophysectomy (Griesbach, Kennedy and Purves, 1941; Purves, 1943; Logothetopoulos and Doniach, 1955). Thyroid cells which are the progeny of a T.S.H.-independent process might show a different reaction from control cells, with regard to tumour formation, in response to prolonged T.S.H. stimulation.

EXPERIMENTAL

The left thyroid lobe was removed and auto-implanted into the subcutaneous fat of the left upper abdomen (*1st implant*) in 50 female and 40 male rats, aged 2½–3 months, from a closed colony of the hooded Lister strain. The operations were done aseptically under ether anaesthesia and were followed by an intraperitoneal injection of 10–25 mg. chloramphenicol suspended in 1 ml. saline, repeated the next day. One half of the females and 25 males were then treated with methylthiouracil given as a saturated solution in the drinking water, made up freshly once a week by suspension of 1 g. crystalline 4-methyl-2-thiouracil per litre of tap water. The remainder (controls) received plain tap water. Three months later, an attempt was made in all the rats to implant the 1st implant again into the subcutaneous fat of the right upper abdomen (*2nd implant*) under similar aseptic precautions. Not all the 1st implants were found; however, some of these were nevertheless recovered in their original site at the end of the experiment. After a further four months an attempt was made to implant the 2nd implants into the subcutaneous fat of the right lower abdomen (*3rd implant*). All the auto-implants were made through a small incision in the skin which was then sutured. The animals

on methylthiouracil were given a break from this toxic drug for one period of 4 weeks and another of 6 weeks during the last 9 months of the experiment. Twenty-seven rats died from intercurrent disease and were discarded. Nine months after the 3rd implantation all the surviving animals were killed by coal gas. The three sites of implantation were searched and all implants removed were fixed, together with most of the residual thyroid lobes, in Helly's fluid. Each thyroid lobe and most of the implants were sectioned at three levels. A few "implants" proved on histology to be lymph nodes or mammary glands and were discarded.

RESULTS

The post-mortem findings are summarised in the Table. Histologically confirmed thyroid implants were recovered in 45 (71 per cent) of the 63 rats which survived the 16 months of the experiment. The numbers are too small for any inferences to be drawn from the higher recovery of implants in females

TABLE.—*Survival of Thyroid Auto-implants in Hemithyroidectomised Rats*

Treatment.	No. of rats.	Sex.	1st implant.	2nd implant.	3rd implant.	Total implants.	Per cent implants recovered.
Methylthiouracil	16	M	3	—	7	10	63
„	17	F	—	—	16	16	94
Nil	12	M	1	2	4	7	58
„	18	F	3	4	5	12	67

Number of implants recovered post mortem. Sixteen months previously the left thyroid lobe was auto-implanted into the subcutaneous tissue of the left upper abdomen (*1st implant*); three months later, re-implantation was attempted into the right upper abdomen (*2nd implant*) and four months afterwards into the right lower abdominal quadrant (*3rd implant*).

and in methylthiouracil-treated rats in each group. The third implants were discrete pale brown subcutaneous nodules 1–2·5 mm. across, somewhat smaller and more fibrous in the control rats. The three first implants in the methylthiouracil-treated animals were larger, 2·5–4 mm. across.

Histology of the implants in all animals showed ovoid nodules most of which were enclosed by a fine fibrous capsule. They contained well formed arteries and veins, groups of thyroid follicles, adipose tissue, scattered haemosiderin-laden phagocytes and frequently a deeply buried regenerated parathyroid remnant (Fig. 2, 3, 5 and 6). There was considerable variation in volume of thyroid and scar tissue.

In the *controls*, the follicles of the implants (Fig. 2 and 3) showed a slight reduction in cell height and increased colloid storage compared with those in the residual lobe in the neck (Fig. 1). With a few exceptions, the 1st and 2nd implants contained more thyroid and less scar tissue than the 3rd implants. One small adenoma was found in the 26 residual thyroid lobes sectioned in the controls and none in the 19 implants.

The residual thyroid lobe in the *methylthiouracil-treated* rats showed the classical effects of prolonged T.S.H. stimulation: diffuse hyperplasia, focal irregularities in follicle appearance and discrete adenomas (Fig. 4). Twenty-six adenomas, varying in diameter from 0·5 to 4 mm., were found in the 29 neck thyroid lobes sectioned in this group. The thyroid tissue in the 3rd implants was more voluminous than in the controls' implants and showed a greater tendency to lobule formation (Fig. 6). The follicles were slightly less hyperplastic than the

lobes in the neck, somewhat irregular in outline and the nuclear arrangement a little haphazard. Focal variations in follicle appearance were rare in the 3rd implants and no definite adenomas were seen in the 23 specimens. However, the 3 first implants showed diffuse hyperplasia and focal irregularities comparable with the residual lobe in the neck, and one small adenoma (Fig. 5).

DISCUSSION

Our findings establish the long term survival of thyroid auto-implants both in control and methylthiouracil-treated hemithyroidectomised rats. In the former the implants showed, on histology, a comparatively inactive state of the follicles ; in the latter, a maintained hyperplastic response. Serial implantation was successful but associated with diminution in size. This might have resulted from mechanical interference with regeneration by scar tissue. In addition, the possibility might be considered that the number of follicles which develop is directly proportional to the number which survive the original implantation process. Since the number of follicles which survive initially is proportional to the surface area of the implant, any failure to re-establish the original volume implanted is bound to lead to a reduced number of surviving follicles on serial implantation.

It has been established (Laqueur, 1949 ; Money and Rawson, 1950 ; Axelrad and Leblond, 1955) that the sequential changes in time in the thyroid associated with prolonged goitrogenic treatment are (1) diffuse hyperplasia, (2) focal irregularities in epithelial hyperplasia, (3) adenoma formation, and ultimately (4) carcinoma induction. Thyroid adenomas are rarely seen before 9 months' treatment but are numerous at 15 months. The histological findings in our methylthiouracil group are typical in the residual lobes and 1st implants of an exposure to goitrogens of over one year. The absence of adenomas and paucity of focal follicular irregularities in the 3rd implants are compatible with goitrogen administration of less than nine months. The 3rd implants were, in fact, done nine months before the animals were killed. Since the implantation led to death of most follicles, the goitrogenic stimulus was a fresh one for the majority of the regenerated follicles established. Also, in the 3rd implants the number of cells which could show neoplasia was smaller. Our results show no greater tendency to tumour formation in implants. However, for the reasons just discussed, no

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EXPLANATION OF PLATE

Photomicrographs of thyroid implants and residual lobes. All stained by H. and E. Magnification × 65 in all.

FIG. 1.—Residual thyroid lobe control male, parathyroid in right upper corner.

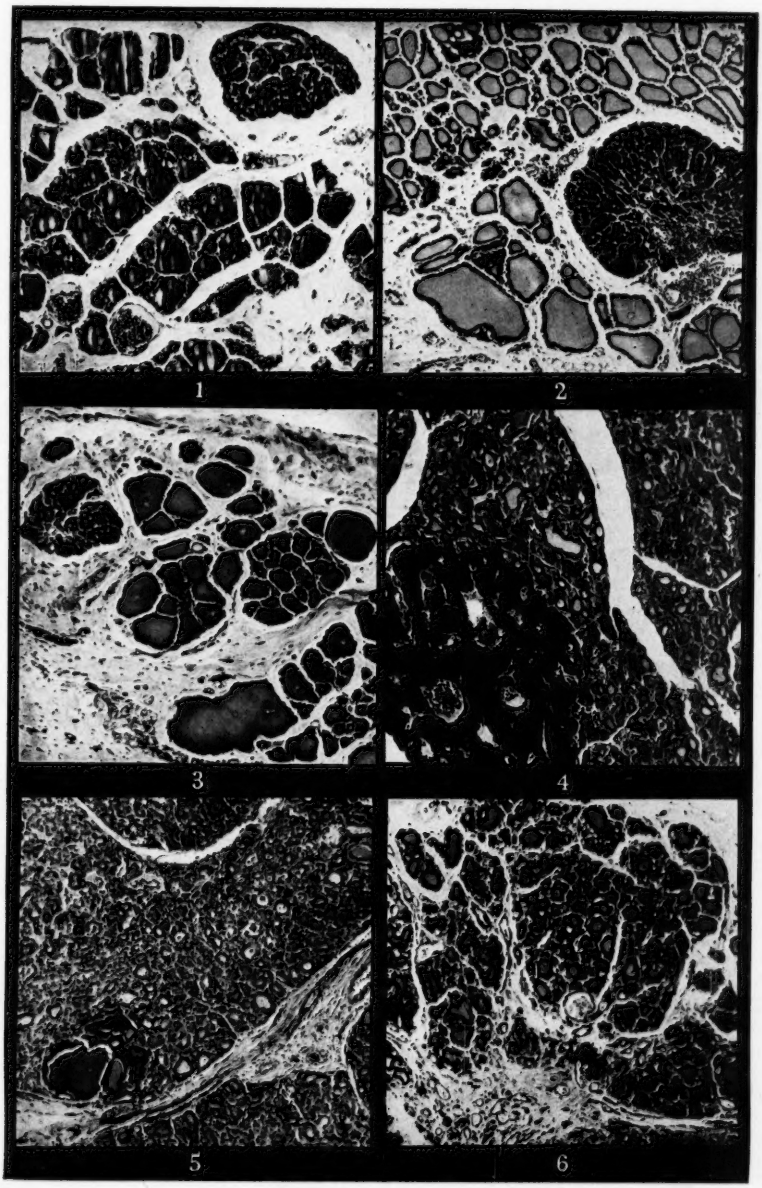
FIG. 2.—First implant control female, embedded parathyroid present, follicles less active than in Fig. 1.

FIG. 3.—Third implant control male, parathyroid in upper left corner, follicles less active than in Fig. 1, more scar tissue than in Fig. 2.

FIG. 4.—Residual thyroid lobe methylthiouracil-treated male, hyperplastic thyroid, large adenoma in left lower half.

FIG. 5.—First implant methylthiouracil-treated male, similar hyperplasia to Fig. 4, small adenoma towards left lower corner.

FIG. 6.—Third implant methylthiouracil-treated male, scar tissue and thyroid tissue ; slightly less hyperplastic than in Fig. 4 and 5,



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firm conclusion can be drawn on this point without repeating the experiment, using single rather than serial implantation and killing the rats after a longer time of goitrogenic treatment.

SUMMARY

The left thyroid lobe was auto-implanted subcutaneously in 90 rats, 50 of which were then maintained on methylthiouracil in the drinking water. Serial re-implantation was attempted in all rats after 3 months and again after a further 4 months. The animals were killed 16 months after the first operation.

Implants were recovered in 26 of the 33 surviving methylthiouracil-treated rats and in 19 of the 30 surviving controls. Seven implants were recovered at the 1st implantation site, 6 at the 2nd, and 32 at the 3rd.

It was established that thyroid auto-implants survive at least 16 months in the hemithyroidectomised rat, in spite of the presence of a residual thyroid lobe in the neck; that they survive serial implantation; that they continue to respond to goitrogenic stimulation.

The implants appeared slightly less active, on histology, than the residual lobes in the neck. Serial implantation led to diminution in size and volume of thyroid tissue and increase in scar tissue. The implants showed no increased tendency to tumour formation.

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